Heat Hardening Capacity of Adult Red Flour Beetles, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) under Acute Thermal Stress

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Heat Hardening Capacity of Adult Red Flour Beetles, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) under Acute Thermal Stress

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ABSTRACT

The red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae), is a cosmopolitan secondary pest of stored grain that exploits cracked kernels and residues, causing serious quality losses and facilitating mycotoxigenic fungal contamination. In Australian bulk storages, the species persists despite phosphine resistance and frequent exposure to surface grain temperatures exceeding 45°C, suggesting rapid thermal plasticity contributes to survival. Heat-hardening capacity was assessed in adults exposed to controlled conditions simulating silo environments. A sublethal threshold of 38°C was identified as the highest acute 1 h exposure permitting ≥75% survival after recovery. Pre-conditioning at this temperature significantly enhanced tolerance: survival at 40°C increased to 73.5% compared with 58.0% in non-hardened cohorts, and at 42°C to 53.5% compared with 29.0%. These results indicate a ~2°C upward shift in tolerance, allowing persistence under field conditions. Reversible immobility at high temperatures supports a plastic, not absolute, survival threshold. Findings are consistent with inducible heat shock proteins, antioxidant systems, and redox regulation underpinning heat hardening in *T.castaneum*. Sublethal exposures in thermally heterogeneous storages may therefore prime survivors, emphasising the need for uniformly lethal heating protocols in postharvest management.

1. Introduction

The red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae), is a cosmopolitan secondary pest of stored products that exploits cracked kernels, milled fractions, and processing residues rather than intact grain. This niche reduces grain quality via frass, fragments, off-odours, and allergens (Hagstrum & Athanassiou, 2019). Post-harvest losses due to stored-product insects are estimated at 9–20% of global yields, with *T.castaneum* recognised as a major contributor; in controlled four-month wheat storage trials, *T.castaneum* alone has caused ≈2% grain weight loss alongside qualitative deterioration (i.e. frass accumulation, odour, reduced baking quality) (Padín et al., 2002; Rault et al., 2024). In Australian postharvest systems, where wheat and other cereals are stored and transported in bulk, *T.castaneum* is among the most frequently detected species in both on-farm and central storages across Queensland, New South Wales, and South Australia. Its populations increasingly harbour both weakly and strongly phosphine-resistant phenotypes (Nayak et al., 2017; Nayak et al., 2021).

At a mechanistic level, resistance is interlinked with broad stress physiology: resistant strains up-regulate detoxification enzymes (cytochrome P450s, GSTs), elevate antioxidant capacity, and remodel mitochondrial metabolism in ways consistent with altered oxidative phosphorylation and diapause-like states (Kim & Lee, 2025; Li et al., 2023). These traits, coupled with behavioural agility in silos, make *T.castaneum* a persistent threat to cereal integrity and marketability in Australia's export-oriented grain economy. The scale of this threat is underscored by ABARES' September 2025 Crop Report, which forecasts national winter crop production at ~62 Mt, including ~33.8 Mt wheat; 22% above the 10-year average (ABARES, 2025). Safeguarding this record crop from postharvest deterioration is therefore directly tied to maintaining both domestic profitability and export access.

Storage microclimates in Australian summers routinely challenge insect physiology. Grain often enters silos at 30–35°C, with surface layers reaching >45°C under solar loading if unaerated (Tilley et al., 2007). Operational disinfestation of empty bins requires ≥50 °C for several hours, yet real-world structures exhibit strong heterogeneity, leaving cooler refugia that insects can exploit. In filled storages, central cores are warmer and drier than peripheries, and field sampling consistently shows beetle aggregation in these zones, likely tracking thermal and fines-rich conditions (Athanassiou & Buchelos, 2020). Despite exposure to nominally lethal extremes, live adults are recovered from unaerated bins, an observation inconsistent with static lethal time estimates and suggesting additional tolerance mechanisms.

The most parsimonious explanation is phenotypic plasticity, particularly heat hardening and short-term acclimation. Heat hardening refers to a rapid inducible increase in tolerance following sublethal exposure. Insects ranging from *Drosophila melanogaster* to *Locusta migratoria* show improved survival and delayed knockdown after mild pre-heating (Arias et al., 2012; Hoffmann et al., 2013; Moghadam et al., 2019). A global meta-analysis of 1,374 effect sizes across 102 species confirmed acclimation plasticity is pervasive but weak, with CT_{max} increasing only ~0.09°C per 1°C acclimation, and juveniles showing greater plasticity than adults (Weaving et al., 2022). Even so, in storage environments where temperatures hover around lethal thresholds, such small increments can determine persistence across daily peaks. Plasticity also interacts with resource states; when combined with nutritional stress, responses are non-additive, broadening survival options in marginal niches (Chakraborty et al., 2025).

Mechanistically, hardening is underpinned by induction of heat shock proteins (HSPs). HSP70, HSP90, and small HSPs (sHSPs) stabilise denaturing proteins, assist refolding, and facilitate degradation of irreparably damaged proteins. Their expression is controlled by heat shock factors (HSFs), modulated epigenetically, and integrated with antioxidant and immune

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pathways (Banfi et al., 2025; Chen et al., 2018). These proteins mitigate heat-triggered reactive oxygen species (ROS), cross-talk with immune signalling, and support cellular integrity during acute stress. In parallel, membrane lipid remodelling preserves bilayer order at high temperature, cytoskeletal adjustments stabilise intracellular structure, and metabolic shifts reallocate energy towards protective functions (González-Tokman et al., 2025; Gunderson et al., 2017; Solano et al., 2025). Together, these responses reduce the accumulation rate of irreversible heat damage.

In *T.castaneum*, transcriptomic data reinforce this model. Sublethal pre-exposures (36–42°C) yield hundreds of differentially expressed genes enriched in HSPs, proteostasis, oxidative defences, and energy metabolism (Lü & Huo, 2018). Knockdown studies confirm causality: silencing hsp18.3 impairs antioxidant responses, reduces fecundity, and elevates thermal mortality, highlighting its role in linking stress tolerance to life-history traits (Xiong et al., 2018). Resistance traits further intersect: phosphine-resistant strains exhibit altered mitochondrial electron transport and redox pathways. Since phosphine toxicity involves lipid peroxidation and ROS generation, resistance mutations (*dld/rph2*, *cyt-b5-r/rph1*) plausibly confer collateral effects on thermal performance via shared oxidative stress circuitry (Shen et al., 2023). Thus, convergent evidence suggests that *T.castaneum* adults can leverage both inducible HSP systems and redox-modulated mitochondrial states to withstand short-term high-temperature insults common in Australian storages.

Two operational features sharpen this inference. First, storage structures are thermally heterogeneous. Beetles can behaviourally thermoregulate by burrowing into fines-rich layers, which heat and cool more slowly, buffering body temperature. Behavioural and physiological buffering together explain why adults persist where air or grain temperatures alone predict mortality (Athanassiou & Buchelos, 2020). Second, structural heat treatments are rarely uniform: while protocols target ≥50°C for hours, cooler pockets inevitably remain. In such refugia, sublethal warming may induce protective hardening instead of mortality, paradoxically priming beetles to survive subsequent peaks. Sublethal exposures raising CT_{max} by fractions of a degree (Weaving et al., 2022) may suffice to sustain populations in these zones.

Against this backdrop, a targeted study of heat hardening in Australian *T.castaneum* populations is warranted. Static lethal time estimates fail to explain field persistence, and while heat hardening is established in other taxa, its operational magnitude and relevance in this beetle under grain-like conditions remain unclear. Two aims were therefore pursued. First, to define the stressful thermal threshold for adults; operationally, the highest acute 1 h exposure allowing ≥75% survival after 24 h recovery. This benchmark aligns with Australian summer storage conditions and provides a practical point of reference for disinfestation strategies. Second, to test whether sublethal pre-treatments at this threshold induce heat hardening, thereby enhancing survival under otherwise lethal exposures. By coupling these organismal outcomes with the species' known HSP biology, mitochondrial redox traits, and field-relevant storage microclimates, the study interrogates whether short-term plasticity enables *T.castaneum* to persist in marginal niches, with direct consequences for postharvest pest management in Australian grain systems.

2. Results

2.1 Calibration of Thermal Equilibrium

Adult *T.castaneum* (10–14 days post-eclosion) were sourced from standardised laboratory colonies (see Supplementary Methods: Population). Prior to experiments, calibration trials were performed to determine how long flour inside 1.5mL Eppendorf tubes required to equilibrate with the surrounding water bath (see Supplementary Methods: Calibration of Thermal Equilibrium). This was critical because beetles are capable of burrowing into the flour, meaning that the flour core - where beetles could remain during exposure - had to reach the programmed bath temperature to ensure consistent stress conditions. Using thermocouple probes, equilibration times ranged from ~32s at 25°C to ~83s at 42°C (Table S1). These times were added to the standard 1 h exposure, ensuring all beetles experienced uniform thermal stress.

2.2 Stressful Threshold (Non-Heat-Hardened Survival)

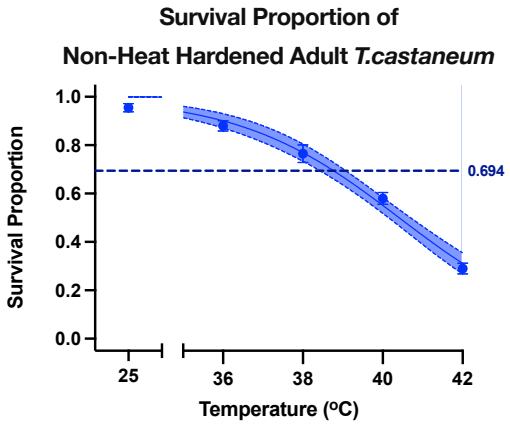

Figure 1. Survival proportion of non-heat-hardened adult *T.castaneum* across acute thermal exposures. Mean survival proportions (±SEM) are plotted for beetles exposed to treatment temperatures ranging from 25–42°C (see Table S2 for replicate data). A nonlinear regression was fitted to the data, constrained between 0 and 1. The horizontal dashed line represents the basal thermal tolerance, calculated as the average survival proportion of non-hardened beetles across test temperatures. The shaded region indicates the 95% confidence interval around the fitted curve.

Baseline survival was determined across five acute exposure temperatures: 25°C (control), 36°C, 38°C, 40°C, and 42°C (20 trials per temperature, 10 beetles per trial; $n = 200$ per treatment, 1,000 beetles total; see Supplementary Methods: Stressful Threshold). Survival remained high at the control (25°C; 0.955 ± 0.017) and 36°C (0.880 ± 0.021), but declined with increasing temperature. At 38°C, survival averaged 0.765 ± 0.036, satisfying the ≥0.75 threshold criterion and establishing 38°C as the stressful sub-lethal temperature. Above this point, survival dropped sharply, with only 0.580 ± 0.025 surviving at 40°C and 0.290 ± 0.022 at 42°C (Table S2; Figure 1).

Behavioural responses further emphasised these thresholds. At the control (25°C), beetles burrowed into the flour, typical of exploratory behaviour. At higher temperatures, beetles instead remained on the surface, often grasping compacted flour particles tightly with their legs. At 40°C and above, many individuals entered a rigid, immobile "catatonic state," clinging to flour masses similarly to how an object might be embraced for stability. Interestingly, some of these beetles later recovered, indicating this behaviour reflected an acute stress response rather than immediate mortality. Together, these data confirm that 38°C is an appropriate sub-lethal stress point, while the overall mean survival across all treatments (0.694) defined the basal thermal tolerance.

2.3 Heat-Hardened Survival

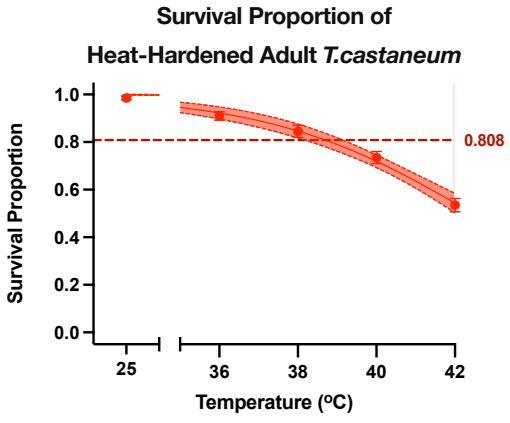

Figure 2. Survival proportion of heat-hardened adult *T.castaneum* to 38°C across acute thermal exposures. Mean survival proportions (± SEM) are plotted for beetles exposed to treatment temperatures ranging from 25–42°C (Table S3). A nonlinear regression was fitted to the data, constrained between 0 and 1. The horizontal dashed line indicates basal thermal tolerance. The shaded region represents the 95% confidence interval around the fitted curve.

To test heat-hardening capacity, beetles were pre-conditioned at 38°C for 1 h plus equilibration (see Supplementary Methods: Heat-Hardened Survival). Groups of 13 beetles were exposed initially, ensuring ≥10 survivors per trial; subsequent assays then tested 10 beetles per replicate across the same five treatment temperatures (20 trials per temperature; *n = 200* per treatment, 1,000 beetles total).

Heat hardening conferred marked survival benefits (Table S3; Figure 2). Survival remained nearly complete at the control (25°C; 0.985 ± 0.008) and 36°C (0.940 ± 0.017). At 38°C, survival was maintained at 0.845 ± 0.026, higher than in non-hardened beetles. Notably, survival at 40°C (0.735 ± 0.025) and 42°C (0.535 ± 0.028) was substantially improved relative to non-hardened counterparts. These results indicate that pre-conditioning at the sub-lethal stress point (38°C) enhanced resistance to otherwise lethal exposures.

2.4 Comparative Analysis of Heat-Hardened versus Non-Heat-Hardened Survival

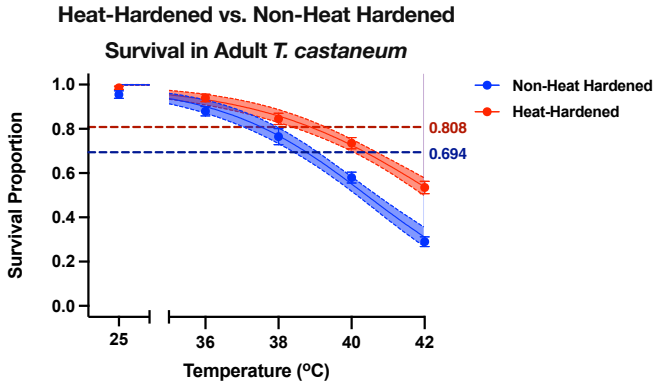

Figure 3. Survival of adult *T.castaneum* under acute thermal stress with and without prior heat hardening. Survival proportions (mean ± SEM) are shown for beetles subjected to 1 h exposures across 25–42°C, assessed 24 h post-exposure. Blue represents non-heat-hardened beetles (Table S2), and red represents heat-hardened beetles pre-treated at 38°C (Table S3). Confidence bands reflect regression fits constrained between 0 (minimum) and 1 (maximum survival). Dashed horizontal lines represent basal thermal tolerance.

Temperature (°C)	F (1,190)	*p* value	Summary
25	1.267	0.3715	ns
36	2.534	0.0748	ns
38	3.378	0.0179	*
40	6.545	<0.0001	****
42	10.35	<0.0001	****

Table 1. Results of pairwise comparisons between heat-hardened and non-heat-hardened adult *T.castaneum* at each treatment temperature. Statistics are reported from Tukey's multiple comparisons test following two-way ANOVA, with F values (1,190), associated p values, and significance summaries (ns = not significant, * = p<0.05, **** = p<0.0001).

Direct comparison of treatments revealed clear differences in thermal resistance (Figure 3). A two-way ANOVA showed significant effects of temperature ($F(4,190) = 177.9$, $p < 0.0001$), treatment ($F(1,190) = 57.9$, $p < 0.0001$), and their interaction ($F(4,190) = 6.68$, $p < 0.0001$). On average, heat-hardened beetles had higher survival (0.808) than non-hardened beetles (0.694). Pairwise comparisons confirmed that heat hardening significantly improved survival at 38°C ($p = 0.0179$), 40°C ($p < 0.0001$), and 42°C ($p < 0.0001$), while no significant differences were observed at the control (25°C) or 36°C (Table 1). Regression fits showed parallel declines in survival with temperature, but hardened beetles consistently shifted upward at higher stress points, effectively extending tolerance by ~2°C compared with non-hardened individuals.

3. Discussion

This study demonstrates that adult *T.castaneum* can extend its upper thermal tolerance through rapid heat hardening. Pre-conditioning at 38°C significantly improved survival at 40°C and 42°C, exposures otherwise associated with sharp mortality in non-hardened individuals. The upward shift in tolerance of ~2°C is modest in absolute terms but ecologically meaningful in storage environments where daily maxima regularly hover around lethal thresholds. Such increments show that small CT_{max} increases can mean the difference between survival and collapse. This capacity helps explain why *T.castaneum* adults are frequently recovered alive from unaerated silos, even when ambient grain layers exceed 45°C (Tilley et al., 2007; Athanassiou & Buchelos, 2020). By showing that sublethal exposures not only spare beetles but actively prime them, the present findings underscore the importance of inducible plasticity in maintaining populations within thermally heterogeneous Australian storage systems.

The survival advantage observed here is consistent with mechanistic foundations of heat hardening in insects. HSPs act as molecular chaperones, stabilising proteins against denaturation, assisting refolding, and targeting irreparably damaged proteins for degradation (Banfi et al., 2025; King & MacRae, 2015). Their expression is strongly induced in *T.castaneum* following sublethal conditioning, with transcriptomic surveys reporting widespread up-regulation of HSPs, proteostasis machinery, oxidative stress defences, and metabolic genes after exposures between 36–42°C (Lü & Huo, 2018). RNAi knockdown of *hsp18.3* produces direct phenotypic consequences - impaired antioxidant capacity, reduced fecundity, and increased mortality under heat stress - demonstrating causality between HSP induction and organismal survival (Xiong et al., 2018). Beyond proteostasis, antioxidant systems and mitochondrial redox pathways buffer against ROS, which accumulate rapidly at high temperature (Chen et al., 2018). Resistant strains of *T.castaneum* exhibit additional metabolic rewiring linked to phosphine resistance, including altered electron transport and lipid peroxidation defences (Chen et al., 2018; Kim & Lee, 2025). These intersections suggest that chemical and thermal stress resilience share overlapping pathways, reinforcing the beetle's capacity to persist in postharvest environments where both challenges routinely co-occur.

Behavioural observations in this study provide further nuance. Beetles exposed to high temperatures often entered rigid "catatonic" states, grasping flour particles tightly before later recovering. Such behaviour indicates that survival is not solely dictated by fixed thermal thresholds but also by reversible suppression of activity, akin to a protective torpor. Comparable phenomena in other insects - termed heat knockdown or heat stupor - are well documented; individuals can enter a reversible coma near CT_{max} and then recover normal activity once temperatures fall (Jorgensen et al., 2020).

From an applied standpoint, these results carry important implications for postharvest pest management. Heat-based disinfestation protocols typically require ≥50°C sustained for several hours to ensure lethality (Tilley et al., 2007). Yet in practice, structural heterogeneity within silos and grain bulks creates cooler refugia where insects experience only sublethal exposures (Athanassiou & Buchelos, 2020). The present data indicate that such exposures may paradoxically prime beetles for enhanced tolerance, rather than weaken them. This narrows the operational margin for thermal treatments: unless heating is uniform and complete, attempts at disinfestation risk producing hardier survivors. Coupled with the widespread presence of weakly and strongly phosphine-resistant phenotypes in Australia (Nayak et al., 2017; Nayak et al., 2021), the plasticity documented here underscores the challenge of managing *T.castaneum* in an export-focused grain economy where quality assurance and market access hinge on maintaining strict phytosanitary standards.

Three avenues of investigation are particularly pressing. First, life-stage dependence must be evaluated. Juveniles in many insects show higher thermal plasticity than adults (Moghadam et al., 2019; Weaving et al., 2022), and *T.castaneum* larvae or pupae may represent more inducible stages than adults. Identifying ontogenetic windows of vulnerability would improve the timing of interventions. Second, the role of population density requires testing. Aggregated beetles in fines-rich silo zones experience altered microclimates and potentially enhanced induction of protective pathways (Đukić et al., 2016). Assessing whether higher densities amplify hardening would clarify how infestation size scales with resilience. Third, potential transgenerational inheritance of heat hardening should be examined. Stress exposure in parents can modify offspring tolerance through epigenetic or maternal effects

(Norouzitallab et al., 2014; Nystrand et al., 2016). If such effects operate in *T.castaneum*, repeated sublethal events could progressively generate more resilient lineages, compounding control difficulties. By addressing these directions, future research can resolve whether inducible plasticity in *T.castaneum* is confined to short-term individual responses or extends across developmental, ecological, and generational dimensions. Such knowledge is critical for refining disinfestation protocols to ensure uniformly lethal exposures.

4. Conclusion

Adult *T.castaneum* exhibit rapid, operationally meaningful heat hardening: a 1-h sublethal pre-exposure at 38°C shifted upper tolerance by ~2°C and substantially improved survival at 40–42°C. This modest physiological increment, coupled with behavioural buffering in fines-rich microhabitats, helps explain persistence in thermally heterogeneous storages where temperatures regularly approach lethal limits. Practically, sublethal warming risks priming survivors; effective heat treatments therefore require uniform, grain-mass-wide lethality rather than hotspot targets. Future work should test life-stage sensitivity, density-dependent microclimates, and transgenerational effects to refine thermal disinfestation protocols and avoid inadvertently selecting for more resilient populations.

References

ABARES. (2025). *Australian crop report: September quarter 2025* (Australian Crop Report, Issue 215). https://www.agriculture.gov.au/abares/research-topics/agricultural-outlook/australian-crop-report

Arias, L. N., Sambucetti, P., Scannapieco, A. C., Loeschcke, V., & Norry, F. M. (2012). Survival of heat stress with and without heat hardening in Drosophila melanogaster: interactions with larval density. *Journal of Experimental Biology*, *215*(13), 2220-2225. https://doi.org/10.1242/jeb.069831

Athanassiou, C. G., & Buchelos, C. T. (2020). Grain properties and insect distribution trends in silos of wheat. *Journal of Stored Products Research*, *88*, 101632. https://doi.org/https://doi.org/10.1016/j.jspr.2020.101632

Banfi, D., Bianchi, T., Mastore, M., & Brivio, M. F. (2025). The Role of Heat Shock Proteins in Insect Stress Response, Immunity, and Climate Adaptation. *Insects*, *16*(7).

Chakraborty, A., Sgrò, C. M., & Mirth, C. K. (2025). Untangling plastic responses to combined thermal and dietary stress in insects. *Current Opinion in Insect Science*, *68*, 101328. https://doi.org/https://doi.org/10.1016/j.cois.2024.101328

Chen, B., Feder, M. E., & Kang, L. (2018). Evolution of heat-shock protein expression underlying adaptive responses to environmental stress. *Mol Ecol*, *27*(15), 3040-3054. https://doi.org/10.1111/mec.14769

Đukić, N., Radonjić, A., Lević, J., Spasić, R., Kljajić, P., & Andrić, G. (2016). The effects of population densities and diet on Tribolium castaneum (Herbst) life parameters. *Journal of Stored Products Research*, *69*, 7-13. https://doi.org/https://doi.org/10.1016/j.jspr.2016.05.007

González-Tokman, D., Villada-Bedoya, S., Hernández, A., & Montoya, B. (2025). Antioxidants, oxidative stress and reactive oxygen species in insects exposed to heat. *Current Research in Insect Science*, *7*, 100114. https://doi.org/https://doi.org/10.1016/j.cris.2025.100114

Gunderson, A. R., Dillon, M. E., & Stillman, J. H. (2017). Estimating the benefits of plasticity in ectotherm heat tolerance under natural thermal variability. *Functional Ecology*, *31*(8), 1529-1539. https://www.jstor.org/stable/48582488

Hagstrum, D. W., & Athanassiou, C. G. (2019). Improving Stored Product Insect Pest Management: From Theory to Practice. *Insects*, *10*(10). https://doi.org/10.3390/insects10100332

Hoffmann, A. A., Chown, S. L., & Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they? *Functional Ecology*, *27*(4), 934-949. https://doi.org/https://doi.org/10.1111/j.1365-2435.2012.02036.x

Jorgensen, L. B., Robertson, R. M., & Overgaard, J. (2020). Neural dysfunction correlates with heat coma and CT(max) in Drosophila but does not set the boundaries for heat stress survival. *J Exp Biol*, *223*(Pt 13). https://doi.org/10.1242/jeb.218750

Kim, D., & Lee, S.-E. (2025). Proteomic evaluation of pathways associated with phosphine-induced mitochondrial dysfunction and resistance mechanisms in Tribolium castaneum against phosphine fumigation: Whole and partial proteome identification. *Ecotoxicology and Environmental Safety*, *289*, 117652. https://doi.org/10.1016/j.ecoenv.2024.117652

King, A. M., & MacRae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Annu Rev Entomol*, *60*, 59-75. https://doi.org/10.1146/annurev-ento-011613-162107

Li, L., Shan, C., Liu, Q., Li, B., & Liu, T. (2023). Comparative Analysis of the Metabolic Profiles of Strains of Tribolium castaneum (Herbst) Adults with Different Levels of Phosphine Resistance Based on Direct Immersion Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry. *Molecules*, *28*(23).

Lü, J., & Huo, M. (2018). Transcriptome analysis reveals heat tolerance of Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae) adults. *Journal of Stored Products Research*, *78*, 59-66. https://doi.org/https://doi.org/10.1016/j.jspr.2018.06.004

Moghadam, N. N., Ketola, T., Pertoldi, C., Bahrndorff, S., & Kristensen, T. N. (2019). Heat hardening capacity in Drosophila melanogaster is life stage-specific and juveniles show the highest plasticity. *Biol Lett*, *15*(2), 20180628. https://doi.org/10.1098/rsbl.2018.0628

Nayak, M. K., Falk, M. G., Emery, R. N., Collins, P. J., & Holloway, J. C. (2017). An analysis of trends, frequencies and factors influencing the development of resistance to phosphine in the red flour beetle Tribolium castaneum (Herbst) in Australia. *Journal of Stored Products Research*, *72*, 35-48. https://doi.org/https://doi.org/10.1016/j.jspr.2017.03.004

Nayak, M. K., Jagadeesan, R., Singarayan, V. T., Nath, N. S., Pavic, H., Dembowski, B., Daglish, G. J., Schlipalius, D. I., & Ebert, P. R.

(2021). First report of strong phosphine resistance in stored grain insects in a far northern tropical region of Australia, combining conventional and genetic diagnostics. *Journal of Stored Products Research*, *92*, 101813. https://doi.org/https://doi.org/10.1016/j.jspr.2021.101813

Norouzitallab, P., Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Vanden Bussche, J., Vanhaecke, L., Sorgeloos, P., & Bossier, P. (2014). Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic Artemia model. *FASEB J*, *28*(8), 3552-3563. https://doi.org/10.1096/fj.14-252049

Nystrand, M., Cassidy, E. J., & Dowling, D. K. (2016). Transgenerational plasticity following a dual pathogen and stress challenge in fruit flies. *BMC Evol Biol*, *16*(1), 171. https://doi.org/10.1186/s12862-016-0737-6

Padín, S., Dal Bello, G., & Fabrizio, M. (2002). Grain loss caused by Tribolium castaneum, Sitophilus oryzae and Acanthoscelides obtectus in stored durum wheat and beans treated with Beauveria bassiana. *Journal of Stored Products Research*, *38*(1), 69-74. https://doi.org/https://doi.org/10.1016/S0022-474X(00)00046-1

Rault, L. C., Morrison, W. R., 3rd, Gerken, A. R., & Bingham, G. V. (2024). Challenges in Assessing Repellency via the Behavioral Response by the Global Pest Tribolium castaneum to Protect Stored Grains. *Insects*, *15*(8). https://doi.org/10.3390/insects15080626

Shen, X., Che, M., Xu, H., Zhuang, X., Chen, E., Tang, P., & Wang, K. (2023). Insight into the molecular mechanism of phosphine toxicity provided by functional analysis of cytochrome b5 fatty acid desaturase and dihydrolipoamide dehydrogenase in the red flour beetle, Tribolium castaneum. *Pesticide Biochemistry and Physiology*, *194*, 105482. https://doi.org/https://doi.org/10.1016/j.pestbp.2023.105482

Solano, L. E., Keshet, U., Reinschmidt, A., Chavez, Y., Hulsy, W. D., Fiehn, O., & Nikolaidis, N. (2025). Dynamic Lipidome Reorganization in Response to Heat Shock Stress. *International Journal of Molecular Sciences*, *26*(7).

Tilley, D. R., Casada, M. E., & Arthur, F. H. (2007). Heat treatment for disinfestation of empty grain storage bins. *Journal of Stored Products Research*, *43*(3), 221-228. https://doi.org/https://doi.org/10.1016/j.jspr.2006.04.005

Weaving, H., Terblanche, J. S., Pottier, P., & English, S. (2022). Meta-analysis reveals weak but pervasive plasticity in insect thermal limits. *Nat Commun*, *13*(1), 5292. https://doi.org/10.1038/s41467-022-32953-2

Xiong, W.-f., Xie, J., Wei, L.-t., Zhang, S.-s., Song, X.-w., Gao, S.-s., & Li, B. (2018). Transcriptome analysis of hsp18.3 functions and regulatory systems using RNA-sequencing in the red flour beetle, Tribolium castaneum. *Journal of Integrative Agriculture*, *17*(5), 1040-1056. https://doi.org/https://doi.org/10.1016/S2095-3119(17)61886-0

Heat Hardening Capacity of Adult Red Flour Beetles, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) under Acute Thermal Stress

SCNC3101 Report

SUPPLEMENTARY INFORMATION

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EXPERIMENTAL METHODS

Population

A laboratory colony of *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) was maintained at the Australian National University, Canberra, within the Wes Whitton Building, between July and September. Beetles were housed in 2 L clear plastic containers (20 × 12 × 12 cm) containing ~600 g of *Laucke multigrain flour* (Laucke Flour Mills, Australia) supplemented with 5% (*w/w*) *Wellness Road* nutritional yeast flakes (Coles, Australia). Each container was closed with a perforated plastic lid, containing multiple small holes (~1 mm diameter) to permit gas exchange while preventing adult escape. The substrate depth was ~3.5 cm, which provided suitable conditions for oviposition, larval development, and pupation. Colony density was maintained at approximately 100–500 individuals per container to ensure robust population turnover without excessive crowding.

Colonies were maintained as overlapping generations, with sexes not separated. Adults, larvae, and pupae were left to develop undisturbed until experiments commenced. Substrate was sieved and refreshed every 4–6 weeks to minimise frass accumulation and microbial growth. For experimental assays, adults were obtained by sieving and standardised to 10–14 days post-eclosion, corresponding to peak reproductive maturity while limiting age-related effects on survival.

Cultures were kept on open shelving inside the insectary room, under natural diel photoperiods programmed to replicate Canberra sunrise–sunset cycles for July–September (~10 h light : 14 h dark to ~12 h light : 12 h dark). No ultraviolet lighting was present above the cultures. The ambient temperature inside the room averaged ~22°C, although seasonal variation was pronounced due to limited air-conditioning control. Additional heat sources from nearby reptile enclosures generated localised gradients of ~23–35°C, while the flour–yeast substrate likely buffered internal container temperatures. Relative humidity was not actively controlled and followed typical seasonal ambient conditions for Canberra winter and spring (~45–70%).

Thermal Sensitivity

Calibration of Thermal Equilibrium

Calibration experiments were first conducted to determine the equilibration time required for flour inside 1.5 mL Eppendorf tubes to reach the target exposure temperatures. For each calibration assay, 1.0–1.6 g of Laucke multigrain flour was weighed into sterile tubes, and a *Type K thermocouple probe* (Omega Engineering HH911T handheld digital thermometer; accuracy ±0.3°C; resolution 0.1°C; NIST traceable calibration) was inserted into the geometric centre of the flour. Tubes were then submerged in a *Benchmark Scientific myBath 2 L Digital Water Bath* (Model B2000-2; accuracy ±0.2°C; uniformity ±0.2°C at 37°C) filled with 1 L of tap water. The water level was maintained above the flour level inside the tubes, ensuring heat conduction through the water rather than slower convection through air. The time required for the internal flour temperature to reach the programmed set point (36, 38, 40, or 42°C) was recorded, and five replicate calibrations were conducted per temperature. The mean equilibration time for each temperature was calculated and added to the standard 1 h exposure duration to ensure that beetles experienced accurate and consistent thermal conditions.

Stressful Threshold (Non-Heat-Hardened Survival)

To determine the stressful temperature threshold, adult beetles were exposed to acute heat stress across a range of temperatures. For each trial, a single adult beetle was placed into a 1.5 mL Eppendorf tube containing 0.10–0.16 g of flour. Flour masses were weighed precisely to two decimal places on an analytical balance to standardise the microenvironment. Tubes were held upright in custom 3D-printed racks designed to secure eight tubes at once; two were used. The racks ensured uniform immersion depth and prevented tube displacement during exposure.

Racks were submerged in 1 L of water in the *Benchmark myBath 2 L Digital Water Bath* at one of five target temperatures: 25°C (control), 36°C, 38°C, 40°C, or 42°C. The water level was maintained so that it exceeded the flour depth inside each tube, ensuring that heating occurred by conduction through the water. Each trial consisted of 10 beetles, and 20 replicate trials were performed per temperature, giving 200 beetles per treatment and 1,000 beetles in total. All temperature treatments were conducted concurrently across five separate water baths to minimise variation in timing. Following exposure for 1 h plus the calibration equilibration time, tubes were removed from the baths and placed on racks at ambient laboratory temperature (~22°C) for a 24 h recovery period prior to mortality assessment.

Mortality Assessment and Survival Proportion

Beetles were assessed for mortality 24 h post-exposure using a two-step protocol. Tubes were first examined visually for spontaneous movement. If no movement was detected, beetles were gently removed with forceps and placed on their dorsal surface to elicit righting behaviour. Individuals that failed to respond were scored as dead. Surviving and dead beetles were transferred to separate post-assay containers to ensure that no beetle was tested more than once.

Survival proportion for each trial was calculated as:

$$Survival\ proportion = \frac{Number\ of\ surving\ beetles}{10}$$

Heat-Hardened Survival

Mean survival proportions across 20 replicates were calculated for each temperature treatment. The stressful threshold was defined as the maximum temperature at which the mean survival proportion was ≥ 0.75. Based on this criterion, 38°C was identified as the stressful threshold, with an average survival proportion of 0.765. To test whether adults could exhibit heat hardening, beetles were first conditioned at the stressful threshold. Groups of 13 beetles were exposed to 38°C (1 h plus equilibration). This elevated number accounted for expected mortality, ensuring that at least 10 surviving beetles were available for subsequent assays. Any surplus survivors were removed and placed in separate holding containers to prevent reuse. After conditioning, beetles were allowed to recover for 24 h at ambient laboratory temperature.

Surviving beetles were then re-exposed under identical assay conditions to five test temperatures (25, 36, 38, 40, and 42°C; 1 h plus equilibration). Mortality was assessed 24 h post-exposure using the same inspection and reflex-righting method described above.

Data Analysis

All analyses were carried out using *GraphPad Prism* (v10.6.0, macOS). The experimental unit was defined as the trial ($n = 10$ beetles per trial), with 20 independent trials conducted for each temperature and treatment condition, resulting in 200 replicate values in total. For each trial, survival was recorded as the number of beetles alive divided by the total number of beetles tested (10), yielding a survival proportion between 0 and 1. These trial-level survival proportions were then used as replicate values for all statistical analyses, ensuring that variation between trials, rather than between individual beetles, was the basis of inference.

For continuous modelling, survival data were analysed using a sigmoidal dose–response regression with a variable slope. The bottom of the curve was constrained to 0 and the top to 1, reflecting the theoretical bounds of proportional survival. Separate curves were fitted for heat-hardened and non-heat-hardened groups. Graphs were produced as XY scatter plots displaying mean survival proportions ± SEM at each temperature. A shaded 95% confidence band was generated around each regression line to visualise the uncertainty of the fitted model. To aid in visualisation, the x-axis was segmented so that the control temperature (25°C) could be plotted in line with the regression curve, which plateaued at higher survival values.

For categorical comparisons, bar graphs were generated showing mean survival proportion ± SEM for each temperature within each treatment group. Brown–Forsythe and Welch ANOVA tests were applied separately to each treatment condition to test for differences in survival across temperatures while accommodating

unequal variances between groups. Post hoc analysis was performed using Dunnett's T3 multiple comparisons, which does not assume equal variance, to identify pairwise differences between temperature treatments.

To test the overall effects of both temperature and treatment, as well as their interaction, a two-way ANOVA was performed. In this model, "row factor" corresponded to temperature and "column factor" to treatment (heat-hardened vs non-heat-hardened). The analysis partitioned variance into three components: temperature effect, treatment effect, and interaction. The significance of each effect was assessed at $\alpha = 0.05$. Where appropriate, mean differences, standard errors, and 95% confidence intervals were reported.

All figures, regressions, and statistical outputs (including ANOVA tables and confidence intervals) were generated within Prism to ensure consistent handling of replicate data.

FIGURES

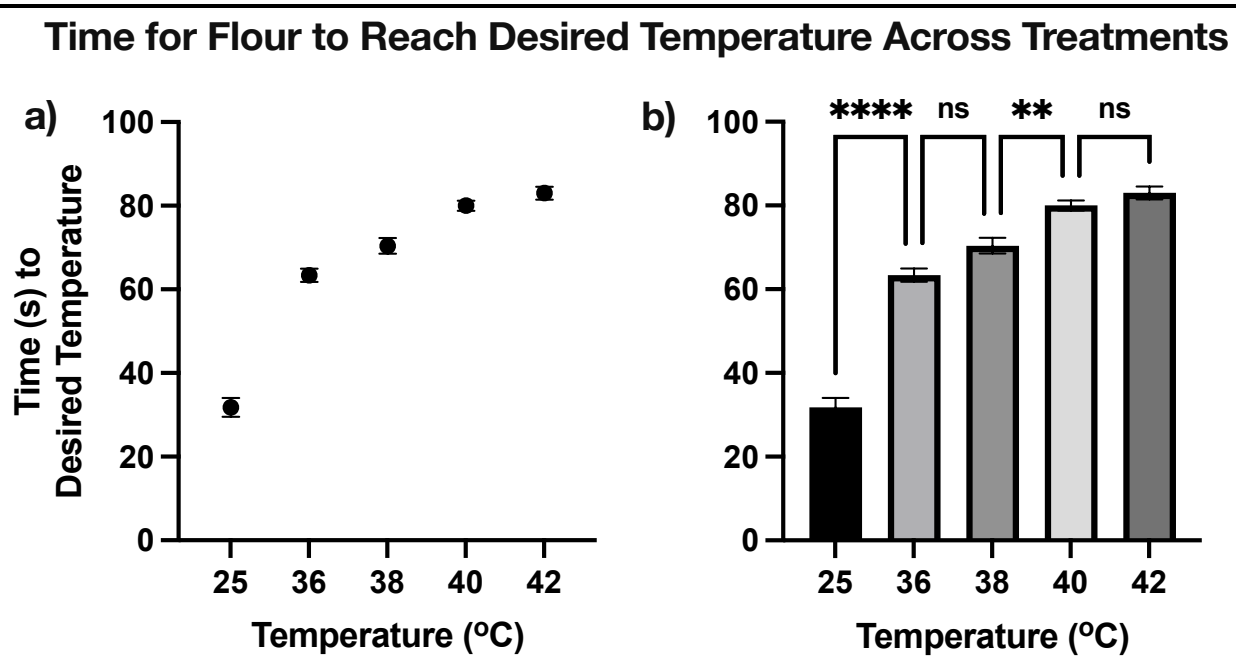

Figure S1. Time required for flour to reach experimental treatment temperatures. (**a**) Mean ± SEM time (s) for flour samples (1.0–1.6 g) inside 1.5 mL Eppendorf tubes to equilibrate to the desired water bath temperature (25–42 °C) as measured by a Type K thermocouple probe. (**b**) Bar graph representation of equilibration times with statistical testing. A one-way ANOVA with Dunnett's T3 multiple comparisons was used to test for differences across temperatures. Significance is indicated as ns = not significant, ** = $p < 0.01$, **** = $p < 0.0001$. Raw replicate values used for these analyses are provided in Table 1.

RAW DATA

Calibration of Thermal Equilibrium

Replicate	25ºC	36ºC	38ºC	40ºC	42ºC
1	27	61	75	77	81
2	29	65	73	78	82
3	31	64	65	84	88
4	40	59	72	80	85
5	32	68	67	81	79
Mean ± SEM	31.8 ± 2.22	63.4 ± 1.57	70.4 ± 1.89	80.0 ± 1.22	83.0 ± 1.58

Table S1. Time (s) required for flour samples (1.0–1.6 g) to reach the target treatment temperature (25, 36, 38, 40, and 42°C) during calibration trials. Values represent five independent replicates per treatment. These calibration measurements were used to standardise equilibration times prior to experimental heat exposures.

Survival Assays in Non-Heat Hardened Beetles

Replicate	25ºC	36ºC	38ºC	40ºC	42ºC
1	1	0.9	1	0.7	0.3
2	0.9	1	0.9	0.7	0.3
3	1	0.9	0.6	0.6	0.4
4	1	0.8	0.7	0.6	0.1
5	0.9	0.8	0.7	0.7	0.2
6	1	1	0.8	0.4	0.2
7	0.8	0.9	0.6	0.5	0.1
8	1	0.9	0.5	0.5	0.3
9	0.8	0.7	1	0.6	0.4
10	1	0.8	0.7	0.7	0.2
12	1	0.9	0.6	0.6	0.3
12	0.8	0.9	1	0.5	0.4
13	1	1	0.8	0.6	0.3
14	1	1	0.9	0.5	0.3
15	1	0.7	0.5	0.5	0.4
16	1	0.9	0.7	0.7	0.3
17	0.9	0.8	0.7	0.5	0.4
18	1	0.8	0.8	0.5	0.3
19	1	0.9	1	0.8	0.2
20	1	1	0.8	0.4	0.4

Table S2. Survival proportions of non-heat-hardened *T.castaneum* adults following 1 h acute exposures across five test temperatures (25, 36, 38, 40, and 42 °C). Each row represents an independent replicate (*n = 20* replicates per temperature, 10 beetles per replicate). Survival was scored 24 h post-exposure using reflex and movement assays.

Survival Assays in Heat-Hardened Beetles

Replicate	25ºC	36ºC	38ºC	40ºC	42ºC
1	1	0.8	1	0.8	0.5
2	1	1	0.6	0.7	0.5
3	1	0.8	0.9	0.6	0.4
4	1	0.9	1	0.8	0.4
5	1	1	0.8	0.7	0.4
6	1	1	0.9	0.8	0.6
7	0.9	0.9	0.8	0.8	0.5
8	1	1	0.9	0.9	0.5
9	1	1	0.8	0.7	0.6
10	1	0.9	0.7	0.5	0.5
12	1	1	0.9	0.8	0.6
12	1	1	0.9	0.8	0.8
13	0.9	0.9	1	0.8	0.5
14	1	1	0.8	0.9	0.4
15	0.9	1	0.8	0.7	0.3
16	1	1	0.7	0.9	0.7
17	1	0.8	0.7	0.6	0.7
18	1	0.9	1	0.7	0.6
19	1	1	0.9	0.6	0.5
20	1	0.9	0.8	0.6	0.7

Table S3. Survival proportions of *T.castaneum* adults subjected to acute thermal exposure following heat hardening at 38 °C. Each replicate consisted of 10 beetles tested individually in 1.5 mL Eppendorf tubes with 0.1–0.16 g flour. Survival was scored 24 h post-exposure at five treatment temperatures (25–42°C). Values represent survival proportions per replicate (*n = 20*).