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Thermal tolerance responses of the two-spotted stink bug, *Bathycoelia distincta* (Hemiptera: Pentatomidae), vary with life stage and the sex of adults

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ABSTRACT

Temperature tolerance is an essential component of insect fitness, and its understanding can provide a predictive framework for their distribution and abundance. The two-spotted stink bug, *Bathycoelia distincta* Distant, is a significant pest of macadamia. The main goal of this study was to investigate the thermal tolerance of *B. distincta* across different life stages. Thermal tolerance indices investigated included critical thermal maximum (CT_{max}), critical thermal minimum (CT_{min}), effects of acclimation on CT_{max} and CT_{min} at 20, 25, and 30 °C, and rapid heat hardening (RHH), and rapid cold hardening (RCH). The Kruskal-Wallis test was used to explore the effects of life stage and acclimation on CT_{max} and CT_{min} and Generalized Linear Models (GLM) for the probability of survival after pre-exposure to RHH at 41 °C for 2 h and RCH at −8 °C for 2 h. CT_{max} and CT_{min} varied significantly between life stages at all acclimation temperatures, but CT_{min} (3.5 °C) varied more than CT_{max} (2.1 °C). Higher acclimation temperatures resulted in larger variations between life stages for both CT_{max} and CT_{min}. A significant acclimation response was observed for the CT_{max} of instar 2 (1.7 °C) and CT_{min} of females (2.7 °C) across acclimation temperatures (20–30 °C). Pre-exposure significantly improved the heat and cold survival probability of instar 2 and the cold survival probability of instar 3 and males. The response between life stages was more variable in RCH than in RHH. Instar 2 appeared to be the most thermally plastic life stage of *B. distincta*. These results suggest that the thermal plastic traits of *B. distincta* life stages may enable this pest to survive in temperature regimes under the ongoing climate change, with early life stages (except for instar 2) more temperature sensitive than later life stages.

1. Introduction

Climatic factors such as temperature and humidity strongly influence insects (Fisher et al., 2021; Jaworski and Hilszczański, 2013). As a result of the ongoing climate change, the intensity and variability of these climatic factors are increasing (Diffenbaugh et al., 2005), resulting in variable temperatures in diel and seasonal cycles (Tarusikirwa et al., 2020). Because insects are ectotherms, their activity and metabolism are greatly influenced by temperature, with high temperature stimulating activity and low temperature suppressing it (Mellanby, 1939). Thus, changes in climatic factors may present physiological challenges and, ultimately, directly impact their thermal fitness (Nyamukondiwa et al., 2018). Therefore, thermal tolerance is of fundamental importance in determining the survival of insects in their distinct microhabitats (Rodrigues and Beldade, 2020).

Phenotypic plasticity is the ability of ectotherms to withstand temperatures outside their optimal zone (Sommer, 2020) through physiological, morphological, and behavioral strategies when introduced to a novel environment (Chidawanyika and Terblanche., 2011; Gray, 2013). Physiologically, insects may respond to temperature variation through processes such as acclimation and hardening (Zhang et al., 2021). Phenotypic plasticity improves the survival of many insect species, including pests such as fruit flies *Ceratitis capitata* Wiedemann, *C. rosa* Karsch (Nyamukondiwa et al., 2010) and codling moths, *Cydia pomonella* Linnaeus (Chidawanyika and Terblanche, 2011). This trait can be evaluated in the laboratory using thermal tolerance indices such as

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critical thermal limits (Motswagole et al., 2019), acclimation (Jumbam et al., 2008), and rapid hardening (Teets et al., 2020).

Critical thermal limits (CTLs) are temperatures at which organisms lose coordinated muscle function after a gradual increase, highest (CT_{max}), or decrease, lowest (CT_{min}) temperature. CTLs are ecologically relevant because they can resemble natural environmental conditions through gradual ramping of temperature (Terblanche et al., 2007). Slow ramping rates (less than 0.5 min^{-1}) are considered more ecological as compared to very slow ramping rates (less than 0.1 min^{-1}), which would result in the hardening of organisms (Escribano-Álvarez et al., 2022; Terblanche et al., 2007; Worland, 2005). CTLs have been used to investigate the thermal fitness, sensitivity, and vulnerability to extreme temperatures of insects (Horne et al., 2019; Nyamukondiwa et al., 2018; Jiranan Piyaphongkul, Pritchard, and Bale 2012). The response pattern of insects to CTLs is decoupled such that there is generally more variation in CT_{min} than CT_{max} (Chanthy et al., 2012; Nyamukondiwa et al., 2010. CTLs may be determined independently or used to quantify the effects of acclimation (Chidawanyika and Terblanche, 2011). Interestingly, the decoupling of CT_{max} and CT_{min} is also evident under acclimation temperatures (Terblanche et al., 2017).

Acclimation is a type of phenotypic plasticity where organisms adjust to changes in environmental conditions. The beneficial acclimation hypothesis (BAH) states an organism previously acclimated to a certain temperature will perform better than a non-acclimated organism (Leroi et al., 1994). Thus, the capacity of an organism to acclimate may be advantageous for coping with climatic variability (Terblanche and Hoffmann, 2020). Beneficial acclimation has been observed in the laboratory (Chidawanyika and Terblanche, 2011; Piyaphongkul et al., 2018) and under field conditions (Thomson et al., 2001). Although the BAH has been criticized (Deere and Chown, 2006; Ramniwas et al., 2020), acclimatory capacity is still being investigated for different taxa (Ruthsatz et al., 2022; Sentis et al., 2022).

Rapid hardening is a quick adaptive phenotypic plasticity with brief pre-exposure to sub-lethal temperatures providing protection from injury while enhancing stress tolerance (Teets et al. 2020). Rapid cold hardening (RCH) initially received considerable attention of insects' ability to survive extreme winter temperatures (Chown and Nicolson, 2004). Rising global average temperatures (Diffenbaugh et al., 2005), have resulted in the increased relevance of rapid heat hardening (RHH) studies of insects (Moghadam et al., 2019; Sørensen et al., 2019; Zhu et al., 2022). These traits can be induced within minutes or hours after exposure to certain sub-lethal temperatures (Nyamukondiwa et al., 2010; Sørensen et al., 2019). For example, a 30-min pre-exposure to sub-lethal temperatures improved the cold survival of fruit flies, *Drosophila melanogaster* Meigen adults (Yi et al., 2007), while the heat survival of the Greenlandic seed bug, *Nysius groenlandicus* Zetterstedt was improved following a pre-exposure of 15–60 min (Sørensen et al., 2019).

Thermal tolerance studies are essential in evaluating insects' thermal sensitivity to temperature changes (Braschler et al., 2021). Thermal tolerance indices have helped build forecast models (Kleynhans et al., 2018; Nyamukondiwa et al., 2013) and management strategies (Nyamukondiwa et al., 2013) of pests. Plastic response of agricultural insect pests is important because it can interact with climate change resulting in increased outbreaks and abundance (Skendžić et al., 2021). Due to pests, there is a projected rise of 25% in yield losses per global degree of mean surface warming for wheat in temperate zones (Deutsch et al., 2018). As a result, the physiological responses of insect pests to temperature have gained renewed recognition as part of integrated pest management (Hallman and Denlinger, 2019). However, temperature response may vary throughout a single life cycle (Marais et al., 2009) and within a generation (Nyamukondiwa and Terblanche, 2010). Anticipating how changing environmental conditions will affect insect pest populations will rely on understanding the thermal tolerance of different life stages (Radchuk et al., 2013; Zhao et al., 2019).

The two-spotted stink bug *Bathycoelia distincta*, Distant is a major endemic pest of macadamia in South Africa and was first detected in 1984 in the Levubu valley situated in the north-eastern corner of the country (Schoeman, 2018). Annual macadamia production has increased from R32 million in 1996 to approximately R5.1 billion in 2021, making it one of the fastest-growing crops in South Africa (Macadamias South Africa NPC, SAMAC, 2021). The annual damage caused by stink bugs has been estimated at up to R200 million (US $15.23 million) (Taylor et al., 2018). *B. distincta* populations are mainly controlled by pesticide application using calendar dates (Schoeman, 2013). However, management strategies for this pest could be further hindered by the ongoing climate change. To date, no systematically gathered thermal tolerance data exists for this species, which is particularly important for integrated pest management. Therefore, the objectives of this study were to 1) quantify CTLs differences between *B. distincta* life stages, 2) evaluate the effects of acclimation on CTLs at different temperatures, and 3) investigate if rapid heat hardening (RHH) and rapid cold hardening (RCH) can enhance survival of different life stages of *B. distincta*.

2. Materials and methods

2.1. Origin of B. distincta and rearing

A colony of *B. distincta* was initiated from eggs that were hand-collected on a commercial macadamia farm between August 2020 and February 2021 in Levubu (23°4′0.96″S, 30°4′31.07″E), Limpopo province, South Africa. Eggs and instars were reared in transparent polypropylene containers (45 × 25 × 30 cm). The newly emerged adults were placed in transparent polypropylene containers (33 cm × 24 cm x 16 cm) for reproduction. All container lids were covered with a fine net and lined with toweling paper on the bottom. Small rounds of cotton moistened with distilled water were randomly placed inside the containers to maintain humidity (Geng and Jung, 2018) and as a source of water for instar 1 nymphs (Dingha and Jackai, 2017). To prevent fungal infection, individuals were transferred to prepared containers with fresh macadamia nuts twice a week. The colony was reared at a temperature of 25 ± 1 °C, 71.8% relative humidity, and a long day photoperiod of 16L: 8D. Temperature and relative humidity were recorded using Thermocron iButtons (Semiconductor Corporation, Dallas/Maxin TX and USA), at hourly intervals for the entire period of rearing.

2.2. CTLs differences between life stages and effects of acclimation at three temperatures

CTLs assays were conducted using a water bath (Grant, SG8 6 GB; Grant Instruments, Cambridge Ltd, UK) filled with 1:1 water: propylene glycol to allow sub-zero temperatures, connected to an insulated system of 11 chambers that enables the fluid to circulate around the chambers. Ten newly emerged individuals per life stage (1–2 days old) were selected and placed individually into the chambers. The temperature was increased at a constant rate of 0.2 °C min^{-1} from 25 to 65 °C for determination of the critical thermal maximum (CTmax) and decreased from 25 to −5 °C for determination of the critical thermal minimum (CTmin) (Fig. 1). To ensure an accurate temperature reading at which individuals reached their CTLs, two Thermocron iButton loggers were inserted into one chamber to record temperature at 1-min intervals. To determine thermal tolerance differences between life stages and changes at three temperatures (20, 25, and 30 °C), individuals were acclimated at 20 and 30 °C (Memmert Peltier-cooled incubator) for 48 h under the photoperiod of 16L: 8D prior exposure to CT_{max} and CT_{min} (Fig. 1). The trials were repeated three times (N = 30) for each life stage. CT_{max} and CT_{min} were identified as the temperature at which an individual loses coordinated muscle function, typically accompanied by loss of movement or neuromuscular control (Nyamukondiwa and Terblanche, 2010). To identify the endpoints of the assays, individuals were prodded gently with a soft paintbrush (Kleynhans et al., 2014).

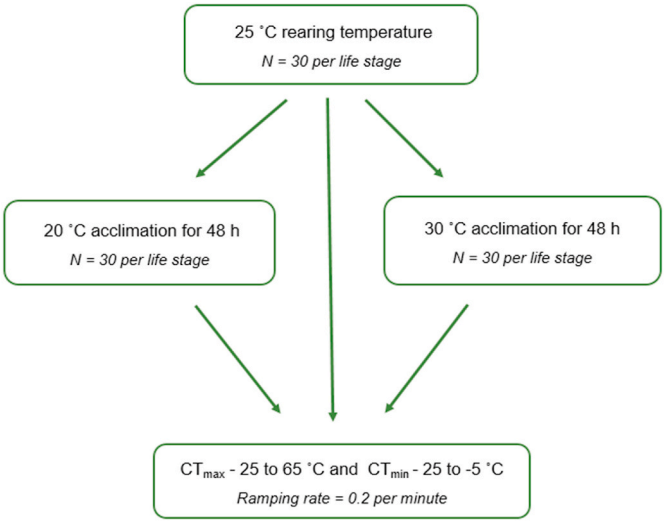

Fig. 1. Schematic diagram of CT_{max} - critical thermal maximum and CT_{min} - critical thermal minimum and effects of acclimation experiments.

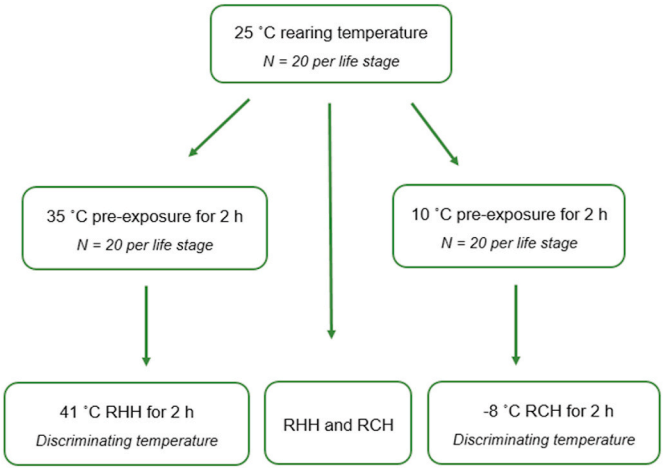

Fig. 2. Schematic diagram of RHH - rapid heat hardening and RCH - rapid cold hardening experiments.

2.3. Determining the discriminating temperature

Discriminating temperature is defined as a temperature that causes approximately 80% mortality (Nyamukondiwa et al., 2010). To establish the discriminating temperature, newly emerged individuals (1–2 days old) were exposed to upper lethal temperatures (ULT) and lower lethal temperatures (LLT). Pilot experiments revealed that temperature and the number of individuals per 60 ml glass vial influenced mortality. Therefore, vials with instars 1 to 3 contained five individuals, and those with instars 4 to adults had two individuals (N = 20). These vials were placed in plastic zip lock bags and exposed to acute temperatures in 1 °C increments ranging from 39 to 43 °C for ULT and −5 to −10 °C for LLT using a standard "plunge" protocol (Terblanche et al., 2008) for 2 h in a programmable water bath. This was achieved by partly submerging the plastic zip-lock bags in the programmable water bath fluid (1:1 water: glycol). Each plastic zip lock bag had two Thermocron iButton loggers recording temperature every minute. Individuals were returned to rearing temperature and survival was recorded as the total number of individuals that responded to stimuli when prodded with a paintbrush after 24 h.

2.4. Rapid hardening

Rapid heat hardening (RHH) and rapid cold hardening (RCH) assays were assessed using the previously described "plunge" protocol. Treatment groups per life stage (N = 20), housed in transparent polypropylene containers (45 × 25 × 30 cm) were transferred from rearing conditions to 35 °C (RHH) and 10 °C (RCH) for 2 h (Nyamukondiwa et al., 2010). Thereafter, the control group and treatment group were exposed to the discriminating temperatures of 41 °C (RHH) and −8 °C (RCH) for 2 h (Fig. 2). Upon completion of each assay, the control and treatment groups were transferred back to the rearing temperature. Similar methods to those described in determining discriminating temperature were used to score survival.

2.5. Statistical analyses

CTLs data per acclimation temperature were tested for normality and homogeneity of variance on the model residuals using the Shapiro-Wilk and Levene tests respectively. Outliers identified as residuals were removed using the cook's distance by removing any values that were three times greater than the mean (Cook, 1977). CTLs did not meet the linear model assumptions and the groups were homoscedastic. The treatment effects (interaction) between acclimation temperatures and life stages was analyzed using the Generalized Linear Model (GLM) assuming a Gaussian distribution and an identity link function with a type III Sum of Squares Anova. Differences between life stages per acclimation temperature were analyzed using the Kruskal-Wallis test and comparisons between life stages were performed using Dunn's post-hoc where significant differences were determined on the alpha level = 0.05 (95% confidence interval) using the ggsignif (Ahlmann-Eltze, 2021) and ggstatsplot R packages (Patil, 2021). The latter tests were used for the effects of acclimation temperatures for each life stage. CTLs of each life stage per acclimation temperature were averaged and the standard deviation, standard error, and 95% confidence intervals were calculated using the Rmisc R package (Hope, 2022). Data were plotted per life stage using the ggplot2 R package (Wickham, 2016) with significant differences indicated by letters above the error bars based on the post-hoc test. To determine the upper (ULT_{80}) and lower (LLT_{80}) lethal temperatures that resulted in 80% mortality, a logistic regression model was fitted to the mortality data per life stage. A mean average for ULT_{80} and LLT_{80} was used as a discriminating temperature for all the stages. A GLM model was fitted to the survival data using a binomial distribution and logit link function to determine the survival probability of *B. distincta* life stages (Marais et al., 2009). All statistical analyses were performed in R, version 4.1.1 (R Core Team, 2022).

3. Results

3.1. CTLs differences between life stages at three acclimation temperatures

Life stages, acclimation temperatures and their interaction explained a significant amount of variation in CT_{max} (GLZ: $\chi^2 = 131.8$, d. f. = 12, $P < 0.001$) and CT_{min} (GLZ: $\chi^2 = 307$, d. f. = 12, $P < 0.001$). Life stage had a significant effect on CT_{max} at 20 °C (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (6) = 77.09, $P < 0.0001$), 25 °C (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (6) = 48.4, $P < 0.0001$), and 30 °C (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (6) = 69.8, $P < 0.0001$). At 20 °C, the multiple comparisons of Dunn's test showed that instars 1 and 2 had a significantly lower CT_{max} than all other life stages (Table 1). At 25 °C, instar 5 and males had significantly higher CT_{max} than the first three instars (Table 1). At 30 °C, the males and instar 2 had a significantly higher CT_{max}, except for instars 4 and 5, while instar 1 was significantly lower than all life stages except for instar 3 and females (Table 1). Similarly, life stage had a significant effect on CT_{min} at 20 °C (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (6) = 124.7, $P < 0.0001$), 25 °C (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (6) = 94.1, $P < 0.0001$), and 30 °C (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (6) = 109.1, $P < 0.0001$). The Dunn's test showed that instars 1 and 2 were

Table 1
Multiple comparisons results of the CT_{max} - critical thermal maximum and CT_{min} - critical thermal minimum (mean ± se) between life stages in response to acclimation at 20, 25, and 30 °C. Means with different letters indicate significant differences per acclimation temperature (Dunn test, $P < 0.05$).

Life stage	20 °C	25 °C	30 °C
CT_{max}			
Instar 1	44.3 ± 0.2^{a}	45.4 ± 0.1^{ac}	44.8 ± 0.1^{a}
Instar 2	44.5 ± 0.1^{a}	45.1 ± 0.1^{a}	46.2 ± 0.1^{b}
Instar 3	45.8 ± 0.2^{b}	45.4 ± 0.1^{ac}	45.2 ± 0.1^{ac}
Instar 4	45.8 ± 0.1^{b}	45.4 ± 0.2^{ab}	46.0 ± 0.1^{b}
Instar 5	45.9 ± 0.1^{b}	46.1 ± 0.2^{bd}	45.9 ± 0.1^{bd}
Female	45.8 ± 0.2^{b}	45.8 ± 0.1^{bcd}	45.4 ± 0.1^{acd}
Male	46.0 ± 0.2^{b}	46.3 ± 0.1^{d}	46.4 ± 0.2^{b}
CT_{min}			
Instar 1	3.6 ± 0.1^{a}	2.7 ± 0.2^{ab}	4.1 ± 0.1^{a}
Instar 2	4.2 ± 0.1^{a}	3.4 ± 0.2^{a}	2.9 ± 0.2^{be}
Instar 3	0.7 ± 0.1^{b}	0.6 ± 0.1^{d}	1.8 ± 0.1^{d}
Instar 4	1.3 ± 0.1^{b}	2.3 ± 0.1^{b}	2.3 ± 0.1^{bd}
Instar 5	1.1 ± 0.1^{b}	2.0 ± 0.1^{bc}	1.6 ± 0.1^{cd}
Female	0.8 ± 0.2^{b}	2.0 ± 0.2^{bc}	3.5 ± 0.1^{ae}
Male	1.1 ± 0.1^{b}	1.6 ± 0.2^{c}	3.0 ± 0.2^{be}

significantly higher than all other stages at 20 °C (Table 1). At 25 °C, instar 3 had the lowest CT_{min}, followed by males, with instar 2 with the highest CT_{min} (Table 1). At 30 °C, instars 3 to 5 had significantly lower CT_{min} than the first two instars, females, and males (Table 1). In general, the response of the life stages to acclimation, measured as the trait effects CT_{max} and CT_{min}, seemed to get more complex with increasing acclimation temperature (Table 1).

3.2. Effects of acclimation on CTLs

Acclimation had a significant impact on the CT_{max} of instar 1 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 21.5, $P < 0.0001$), instar 2 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 45.5, $P < 0.0001$), instar 3 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 7.3, $P = 0.03$), and instar 4 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 9.03, $P = 0.01$), and females (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 6.5, $P = 0.04$) while instar 5 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 0.89, $P = 0.6$), and males (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 3.7, $P = 0.2$) did not differ across acclimation temperature (Fig. 3a). Dunn's test multiple comparisons showed that the CT_{max} of instar 1 increased by 1.1 °C from 20 to 25 °C and decreased by 0.6 °C from 25 to 30 °C. The CT_{max} of instar 2 increased by 0.9 °C from 20 °C to 25 °C, by 1.1 °C from 25 to 30 °C, and by 1.7 °C from 20 to 30 °C. Instar 3 CT_{max} decreased by 0.6 °C from 20 to 30 °C. The CT_{max} of instar 4 increased by 0.6 °C from 25 to 30 °C (Fig. 3a). In response to CT_{min}, acclimation had a significant impact on all life stages; instar 1 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 26.4, $P < 0.0001$), instar 2 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 19.8, $P < 0.0001$), instar 3 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 30.6, $P < 0.0001$), instar 4 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 25.8, $P < 0.0001$), instar 5 (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 15.9, $P < 0.0001$), female (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 42.5, $P < 0.0001$), and male (ANOVA: $\chi^2_{kruskal\text{-}Wallis}$ (2) = 37.2, $P < 0.0001$) (Fig. 3b). The CT_{min} of instar 1 decreased from 20 to 25 °C by 0.9 °C and increased by 1.4 °C from 25 to 30 °C. The CT_{min} of instar 2 increased by 0.8 °C from 20 to 25 °C and from 20 to 30 °C it decreased by 1.3 °C. Instar 3 CT_{min} increased by 1.1 °C from 20 to 30 °C and also increased by 1.2 °C from 25 to 30 °C. The CT_{min} of instar 4 increased from 20 to 25 and 30 °C by 1 °C. The CT_{min} of instar 5 increased by 0.9 from 20 to 25 °C and decreased by 0.4. The CT_{min} of females increased from 20 to 25 °C by 1.2 °C, from 25 to 30 °C by 1.5 °C, and from 20 to 30 °C by 2.7 °C. The CT_{min} of males increased from 20 to 25 and 30 °C by 0.5 and 1.9 respectively (Fig. 3b).

3.3. Discriminating temperatures

After exposure to acute temperatures for 2 h, 80% mortality for each life stage ranged from 40.4 to 41.5 °C for ULT_{80} and -7.7 to −9.5 °C for LLT_{80} (Table 2, Fig. 4a and b). ULT_{80} and LLT_{80} of all life stages were averaged and used as the discriminating temperatures for rapid hardening assays.

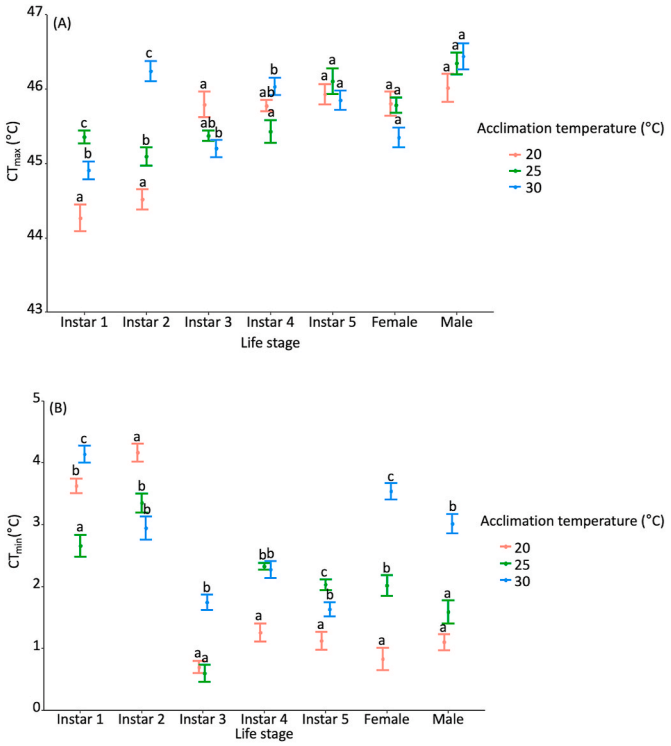

Fig. 3. Effects of acclimation at three temperatures (mean ± se) in response to CT_{max} - critical thermal maximum (A) and CT_{min} - critical thermal minimum (B). Letters indicate significant differences between temperatures for each life stage.

3.4. Rapid hardening

Pre-exposure had a significant impact on heat survival ($P < 0.001$) of all *B. distincta* life stages (Table 3). However, the survival probability of all life stages except for the females decreased relative to instar 1. (Table 3, Fig. 5a). In response to RCH, pre-exposure also had a significant impact on cold survival ($P < 0.001$) (Table 3). The survival probability of all life stages decreased relative to instar 1 (Table 3, Fig. 5b). Among all life stages, pre-expose improved both the heat and cold survival probability of instar 2 (Fig. 5a and b).

4. Discussion

Thermal tolerance varied significantly with *B. distincta* life stages and sex. The variation between life stages increased with increasing acclimation temperatures. The results were more pronounced for colder temperatures for both CTLs and rapid hardening. However, the

Table 2
Logistic regression results for the ULT - upper lethal temperature and LLT - lower lethal temperature (mean ± 95% confidence intervals) that resulted in 80% mortality for each life stage after exposure to acute temperatures for 2 h.

Life stage	ULT_{80} (°C)	LLT_{80} (°C)
Instar 1	41.4 ± 1.2	−9.0 ± 1.0
Instar 2	40.4 ± 1.3	−7.9 ± 1.2
Instar 3	41.2 ± 0.3	−8.5 ± 0.9
Instar 4	41.1 ± 0.9	−9.5 ± 1.5
Instar 5	41.0 ± 1.6	−8.7 ± 0.9
Female	41.4 ± 0.8	−7.8 ± 1.1
Male	41.5 ± 0.8	−7.7 ± 0.9

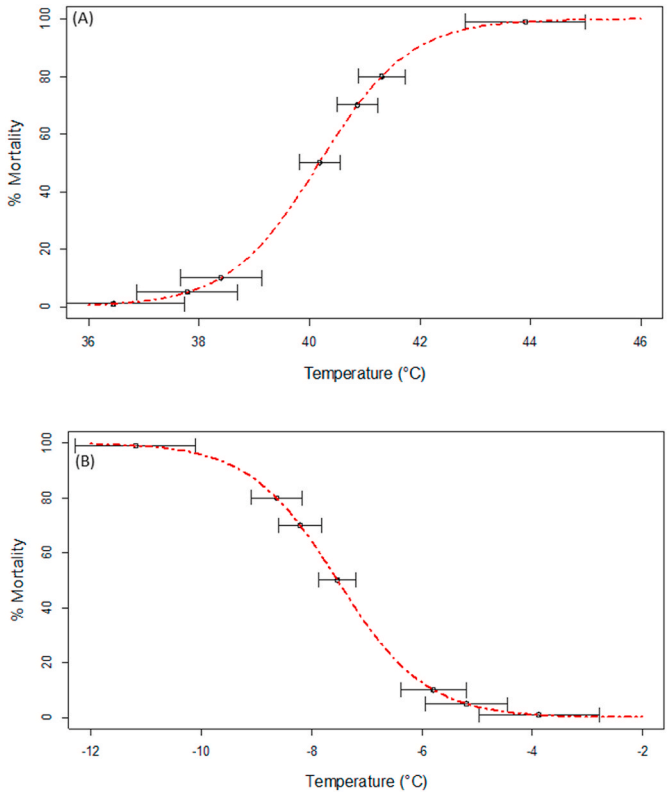

Fig. 4. Fitted logistic regression for temperatures that caused 10–99% mortality of *B. distincta* life stages (nymphal and adults) for the determination of UDT - upper discriminating temperature (A) and LDT - lower discriminating temperature (B). Error bars represent 95% confidence intervals on the discriminating temperatures.

divergence between life stages was less for rapid hardening compared to CTLs. Thermal plasticity for CT_{max} and rapid hardening peaked in instar 2 while females were the most plastic for CT_{min}. Instar 1 was identified as the most temperature-sensitive while later life stages generally were more stress-resistant than earlier life stages.

4.1. CTLs differences between life stages at three temperatures

Thermal tolerance variation of *B. distincta* is consistent with that observed between life stages of other taxa such as the kelp fly, *Paractora dreuxi* Séguy (Marais et al., 2009) and *Chilo partellus*, Swinhoe (Mutamiswa et al., 2019). Instar 1 was heat-intolerant among all other life stages and this is similar to the instar 1 nymphs of the Brown Planthopper, *Nilaparvata lugens* Stål (Piyaphongkul et al., 2012). Dehydration is a major factor determining survival, and improvement in evaporative cooling may have resulted in heat resistance in larger-bodied later life stages relative to instar 1 (Chown et al., 2011; Johnson and Stahlschmidt, 2020;Le Lann et al., 2011). Different species of ants also showed higher CT_{max} in relation to their larger bodies (Baudier et al., 2015; Johnson and Stahlschmidt, 2020). Extreme environmental temperatures can therefore pose a threat to smaller-bodied individuals (Piyaphongkul et al., 2012). Consequently, instar 1 of *B. distincta* will be the most susceptible to heat waves.

In contrast, instar 1 nymphs of Jack Beardsley mealybug, *Pseudococcus jackbeardsleyi* Gimpel and Miller (Piyaphongkul et al., 2018), and field-collected third instar larvae of kelp fly, *Paractora dreuxi* (Klok and Chown, 2001) had significantly higher CT_{max} than the adults. Additionally, the immobile pupal stage of the tropical butterfly life cycle, *Bicyclus anynana* Butler was more heat tolerant than other life stages (Klockmann et al., 2017). In early life stages, mobility may be absent or limited, resulting in a lack of alternative behavioral strategies. This would then make early life stages more resistant to environmental temperature changes (Bowler and Terblanche, 2008). Instar 1 of *B. distincta* remains on egg clusters after hatching and the lower CT_{max} could be related to the cooler microhabitats provided by macadamia trees. Additionally, instar 1 does not feed (Rivera and Mitchell, 2020) as the energy gained from feeding may influence physiological tolerance (Hofmann and Todgham, 2010; Rogers et al., 2021).

The CT_{max} (44.3–46.4 °C) was less variable between life stages than CT_{min} (0.6–4.1 °C). Between the nymphal life stages of Dubai cockroach, *Blaptica dubia* Serville, the CT_{max} ranged from 44. 8–49.9 °C, while the

Table 3
Results of the Generalized Linear Model (GLM) with a binomial distribution and logit link function for the effects of RHH - rapid heat hardening and RCH - rapid cold hardening on the survival of *B. distincta* life stages after pre-exposure for 2 h.

Life stage	Estimate	Std. Error	Z value	P value
RHH				
Intercept	2.71	3.63	0.74	0.45
Pre-exposure	1.00	2.62	3.84	**<0.001** ***
Instar 2	−1.55	4.92	−3.14	**<0.001** **
Instar 3	−7.75	4.78	−1.62	0.10
Instar 4	−4.54	4.78	−0.94	0.34
Instar 5	−1.18	4.86	−0.24	0.80
Female	2.51	5.02	0.50	0.61
Male	−2.17	4.90	0.00	1.00
Survival probability $= \frac{e^{2.72+Pre-exposure\ temperature-1.55Instar2-7.75Instar3-4.54Instar4-1.18Instar5+2.51Female-2.17Male}}{1+e^{2.72+Pre-exposure\ temperature-1.55Instar2-7.75Instar3-4.54Instar4-1.18Instar5+2.51Female-2.17Male}}$				
RCH				
Intercept	1.02	0.43	2.35	0.01 *
Pre-exposure	1.34	0.26	5.01	**<0.001** ***
Instar 2	−1.69	0.54	−3.10	**0.001** **
Instar 3	−1.80	0.54	−3.30	**<0.001** ***
Instar 4	−1.12	0.54	−2.04	0.04 *
Instar 5	−2.03	0.54	−3.69	**<0.001** ***
Female	−2.14	0.55	−3.89	**<0.001** ***
Male	−2.26	0.55	−4.08	**<0.001** ***
Survival probability $= \frac{e^{1.02+1.34Pre-exposure\ temperature-1.69Instar2-1.80Instar3-1.12Instar4-2.03Instar5-2.14Female-2.26Male}}{1+e^{1.02+1.34Pre-exposure\ temperature-1.69Instar2-1.80Instar3-1.12Instar4-2.03Instar5-2.14Female-2.26Male}}$				

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

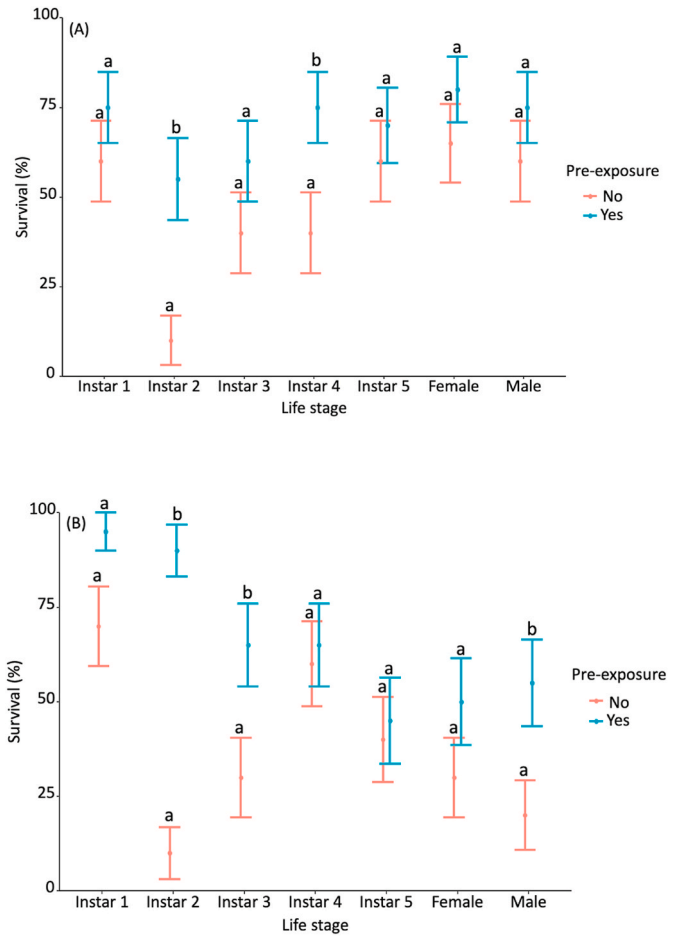

Fig. 5. Survival (mean ± se) of *B. distincta* life stages in response to RHH - rapid heat hardening (A) and RCH - rapid cold hardening (B) after exposure to discriminating temperatures of 41 and -8 °C for 2 h, respectively.

CT_{min} ranged from −2 to −3.1 °C (Wu et al., 2017). Contrastingly, the CT_{max} of the bumblebee, genus *Bombus* Latreille was more variable than the CT_{min}, ranging from 42 to 65 °C and 1.4–8 °C, respectively (Oyen and Dillon, 2018). Observed CTLs for *B. distincta* are comparable with those recorded previously for other laboratory-reared insects with larger variation in CT_{min} and less variation in CT_{max} (Käfer et al., 2020; Klok and Chown, 2001). The remarkably limited plasticity in response to high temperatures could result from the irreversible cell damage caused by water loss as the temperature increases (Hallman and Denlinger, 2019). Klok et al., (2004) noted that fewer physiological factors contribute to heat tolerance than cold tolerance leading to more variation in CT_{min}. For example, tolerance to high temperatures is mainly controlled at cellular levels, whereas low tolerance can be improved by increasing poly cryoprotectants that prevent body fluids from crystallization (Sinclair et al., 2003). Variations in CT_{max} and CT_{min} may also result from the methodology used (Terblanche et al., 2007). Using different ramping may yield different results (Terblanche et al., 2008). However, the current study used a slower ramping rate of 0.2 min^{-1} because of its ecological relevance (Escribano-Álvarez et al., 2022). Furthermore, Chown et al., (2009) and Terblanche et al., (2006) showed that slower ramping rates may result in more plasticity, though the opposite might be the case for other organisms (Terblanche et al., 2007).

Poor cold tolerance of instars 1 and 2 indicates that they cannot cope with gradual temperature decreases compared to other stages. The latter is not surprising as *B. distincta* is more abundant in summer when macadamia nuts are available and declines in winter during postharvest (Schoeman, 2018). While scouting surveys have detected both nymphs and adults in winter (Schoeman and Mohlala, 2012), this study did not specify which nymphal stages were present. However, as macadamia nuts begin to develop in early summer, only adults return to the macadamia orchards (Fourie et al., 2022; Schoeman and Mohlala, 2012). Consequently, the cold tolerance variation between the life stages of *B. distincta* reflects the seasonal timing of developmental stages.

4.2. Effects of acclimation on CTLs

The effects of acclimation on CTLs have been shown to differ between life stages and the magnitude of acclimation temperatures (Mutamiswa et al., 2019). The latter is consistent with the differences observed between the life stages of *B. distincta* across acclimation temperatures for both CT_{max} and CT_{min}. However, acclimation revealed a high level of plasticity in cold tolerance in contrast to heat tolerance between life stages. Except for the CT_{max} of instar 2 and CT_{min} of females, minor acclimation effects in response to CTLs were also reported for the bumblebees, genus *Bombus* (Oyen and Dillon, 2018) and beetles, *Zygogramma bicolorata* Pallister (Chidawanyika et al., 2017). Weak responses to acclimation might suggest that most life stages of *B. distincta* compensate for environmental variations behaviorally.

B. distincta starts feeding with the emergence of instar 2, which could have led to improved tolerance of this stage through its nutritional status. In addition, instar 2 is darkly pigmented, which has been shown to absorb more heat (Stuart-Fox et al., 2017). Thus, this stage may have had an advantage in acclimating faster than other life stages because of its smaller body size resulting in a more robust response to acclimation. Rohr et al. (2018) highlighted the relationship between acclimation duration and organismal body size. This study suggested that short acclimation periods might favor smaller organisms over larger ones as they may have time to fully acclimate because of their body size.

In contrast, female' CT_{min} improved across acclimation temperatures implying that they may respond better to environmental cues. *B. distincta* adults may be expected to show more plastic responses to cold temperatures as they go through diapause in winter (Schoeman and Mohlala, 2012). Indeed, a study of diapause in the seasonal cycle of stink bugs from the temperate zone confirmed that most bugs overwinter as adults surviving low temperatures through freeze avoidance (Saulich and Musolin, 2012). Diapause has been shown to improve cold tolerance of the brown marmorated stink bug, *Halyomorpha halys* Stål both in laboratory and field conditions (Cira et al., 2018). Therefore, stink bug adults may be cold resistant due to the low temperatures they encounter while overwintering. Adults of the southern green stink bug, *Nezara viridula* Linnaeus were also cold-tolerant after a seven-day acclimation period (Chanthy et al., 2012). Thus, diapause may be an important factor in determining cold tolerance of stink bug adults under extremely low temperatures.

The physiological mechanisms underlying enhanced stress tolerance might be linked to the production of heat shock proteins activated under stress to protect against the denaturation of proteins under extreme temperatures (Farahani et al., 2020; Harvey et al., 2020). The upregulation of heat shock proteins is exhibited in response to heat and cold tolerance and has been shown to differ among insect life stages (Rinehart et al., 2006; Teets et al., 2019). The larvae of the flightless midge, *Belgica antarctica* Jacobs produced more heat shock proteins than adults under temperature stress (Rinehart et al., 2006). Similarly, heat and cold tolerance of the developmental life stages of the carob moth larvae, *Ectomyelois ceratoniae* Zeller was confirmed to be associated with the upregulation of two heat shock proteins (HSP70 and HSP90) (Farahani et al., 2020). Thus, some life stages of *B. distincta* (instar 2 and females) could upregulate heat shock protein synthesis under stress better than other stages depending on the intensity of the temperature.

4.3. Rapid hardening

The lack of plastic response to RHH found here was also the case for

the fruit flies, *Ceratitis capitata and Ceratiti rosa* (Nyamukondiwa et al., 2010). This outcome may be owing to the time interval (2 h) used to induce rapid hardening for *B. distincta* life stages. Different time intervals and pre-exposure temperatures prior to heat knockdown have effects on survival (Marais et al., 2009; Nyamukondiwa et al., 2010; Pieterse et al., 2017). A short-time interval of 45 min successfully induced RHH in the Greenlandic seed bug, *Nysius groenlandicus* (Sørensen et al., 2019), while a 2 h time interval and different temperatures resulted in poorer RHH response in fruit flies (Nyamukondiwa et al., 2010). Short-time intervals have also been shown to be beneficial in improving the survival of *Drosophila* (Hoffmann et al., 2003). Using the same time interval (2 h) and pre-exposure temperature (35 °C) as in the current study, RHH was successfully induced for the western flower thrips, *Frankliniella occidentalis* Pergande (Li et al., 2011). Therefore, this could mean that environmental temperatures of *B. distincta* should be considered when determining the pre-exposure temperature rather than using the time intervals of other insects from the literature.

The complex response of *B. distincta* life stages to RCH is evident in other taxa, with considerable variation between life stages (Lee et al., 2006; Terblanche et al., 2007). RCH has also decreased with age (Czajka and Lee, 1990) and corresponded with what was observed for *B. distincta* life stages. In contrast, adults of the kelp fly *Paractora dreuxi* did not respond to RCH (Terblanche et al., 2007). Life stages undergo multiple stressors associated with seasonal changes during development, which explains the variable response to extreme temperatures (Chown and Terblanche, 2006). While possible mechanisms for RCH remains poorly understood and disputed among authors, an increase in hemolymph osmolarities, changes in phospholipid composition and production of cold-induced proteins and polyols play an essential role (Koštál et al., 2001) but varies between species (Chown and Terblanche, 2006).

Overall, CT_{max} of *B. distincta* life stages was considerably higher than temperatures observed for RHH, while RCH was much lower (>5 °C) than CT_{min}. CTLs and rapid hardening represent different aspects of the thermal tolerance indices (Terblanche et al., 2011). CTLs experiments took more than 2 h to complete, which could have given individuals enough time to harden, resulting in higher CT_{max} and may explain the significant variation between CTLs and rapid hardening. The ecological implications of the differential thermal tolerance of *B. distincta* to CTLs and rapid hardening require further investigation because the underlying mechanisms are still poorly understood. These results demonstrate the effects of temperature, developmental stage, and sex on thermal tolerance. The methodological approach used may have affected the obtained results because no prior experiments (i.e., testing of different ramping rates, time intervals, and temperature for rapid hardening) were conducted due to the small size of the colony. Consequently, ramping rates for CTL and time intervals for rapid hardening assays were adopted from the literature. Importantly, *B. distincta* was reared on a host plant eliminating the effects of an artificial diet on the development (Dingha and Jackai, 2017) and, ultimately, thermal tolerance.

Finally, there seems to be more plasticity in response to cold tolerance, particularly for instar 2 and adults. *B. distincta* life stages perform better in response to gradual increases than temperature decreases, but they perform worse when exposed to sudden increases in temperature. Thus, high mortality rates in *B. distincta* populations may be expected with sudden extreme high temperatures and gradual temperature decreases. In contrast, sudden temperature decreases could improve survival at even colder temperatures. In the field, *B. distincta* is exposed to temperatures that are close to their thermal tolerances estimates. The mean average temperature recorded from October 2018 to March 2022 was 20.48 °C with a maximum of 41.95 °C and a minimum of 1.1 °C. The highest and lowest CT_{max} recorded were 46.4 and 44.3 °C, and for CT_{min} were 4.1 and 0.6 °C, respectively. In hardening responses, *B. distincta* life stages survived 41 and -8 °C for 2 h. CTLs and rapid cold hardening estimates exceeded the maximum and minimum ambient temperatures inside and outside the macadamia orchards. In conclusion, this study shows that the thermal plastic traits of *B. distincta* life stages coupled with behavioural strategies may enable this pest to survive temperature changes associated with the ongoing climate change, with instar 2 and adults being temperature resistant and early life stages temperature sensitive.

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CRediT authorship contribution statement

Mulalo M. Muluvhahothe: Writing – original draft, Formal analysis. **Elsje Joubert:** Writing – review & editing, Formal analysis. **Stefan H. Foord:** Writing – review & editing, Formal analysis, Contributions from all authors were incorporated into the paper.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

CTLs, lethal, and hardening data are deposited in the Mendeley data repository (https://data.mendeley.com/research-data/.)

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