

Ontogenetic responses of physiological fitness in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in response to repeated cold exposure

Research Paper

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Abstract

In this era of global climate change, intrinsic rapid and evolutionary responses of invasive agricultural pests to thermal variability are of concern given the potential implications on their biogeography and dire consequences on human food security. For insects, chill coma recovery time (CCRT) and critical thermal minima (CT_{min}), the point at which neuromuscular coordination is lost following cold exposure, remain good indices for cold tolerance. Using laboratory-reared *Spodoptera frugiperda* (Lepidoptera: Noctuidae), we explored cold tolerance repeated exposure across life stages of this invasive insect pest. Specifically, we measured their CT_{min} and CCRT across four consecutive assays, each 24 h apart. In addition, we assessed body water content (BWC) and body lipid content (BLC) of the life stages. Our results showed that CT_{min} improved with repeated exposure in 5th instar larvae, virgin males and females while CCRT improved in 4th, 5th and 6th instar larvae following repeated cold exposure. In addition, the results revealed evidence of cold hardening in this invasive insect pest. However, there was no correlation between cold tolerance and BWC as well as BLC. Our results show capacity for cold hardening and population persistence of *S. frugiperda* in cooler environments. This suggests potential of fall armyworm (FAW) to withstand considerable harsh winter environments typical of its recently invaded geographic range in sub-Saharan Africa.

Introduction

Repeatability or reproducibility experiments are profound tools that were originally developed for independent testing of the precision of experimental protocols. In biological research, the repeatability of observational data can be used to track organismal plastic and genetic responses to stress factors at the individual or population level at various temporal scales (Averages-Weber *et al.*, 2015; Niemelä and Dingemanse, 2017; Näslund, 2021). Given the escalated attention on climate change in recent years, repeatability studies (though controversial) can be pivotal in investigating basal and plasticity of thermal tolerance (Morgan *et al.*, 2018; O'Donnell *et al.*, 2020; O'Neill *et al.*, 2021) where both environmental and genetic phenotypic variation effects can be used to determine within-individual trait variability (Grinder *et al.*, 2020). If the thermal tolerance of a tested organism is consistent over time, denoting high repeatability, it indicates that the adaptive potential of the trait is high while the converse is true for low repeatability (Morgan *et al.*, 2018).

For insects, body temperature depends on ambient conditions mediating biochemical and physiological processes therein (Chown and Nicolson, 2004; Sinclair *et al.*, 2015). Subsequently, such organismal responses mediate development and can cascade to population level through factors such as seasonality, geographic distribution and voltinism (Du Plessis *et al.*, 2020; Phophi *et al.*, 2020; Tarusikirwa *et al.*, 2020; Nyamukondiwa *et al.*, 2022). Of interest is how the magnitude and frequency of thermal extremes in the form of heat waves and cold snaps wrought by the changing climates influence pest physiology, survival and key life-history traits (Tollefson, 2014) as it has direct implications on their population dynamics (Chidawanyika *et al.*, 2012, 2020) and ultimately food security (Gregory *et al.*, 2009). Thus, apart from magnitude of thermal exposure, insects experience different mode of thermal fluctuations (e.g. acute vs. chronic, rapid vs. slow fluctuations and/or repeated exposures) typical of diel and seasonal changes (Colinet *et al.*, 2007). Such extremes, and not average temperatures drive several organismal responses including evolutionary adaptations within and across generations (Cox *et al.*, 2010; Travis 2014; Buckley and Huey, 2016) and define geographic ranges via various demographic tipping points (Lynch *et al.*, 2014).

Indeed, insects have evolved diverse morphological, physiological and behavioural adaptations to withstand and colonize otherwise lethal novel environments (Bale, 2002; Neal *et al.*, 2021). For example, overwintering insects are known to survive stressful low temperatures through employing cold tolerance strategies such as rapid cold hardening (RCH), freeze tolerance and freeze avoidance (Sinclair *et al.*, 2015; Feng *et al.*, 2018). Freeze-tolerant insects survive intracellular ice formation through use of cryoprotectants, removal of ice nucleators and anti-freeze heat shock proteins synthesis (Elnitsky *et al.*, 2008; Storey and Storey, 2012; Toxopeus *et al.*, 2019). On the contrary, freeze-intolerant/avoidant insects cannot withstand internal ice formation but survive through keeping their body fluids under a supercooled condition (Sinclair *et al.*, 2015; Andreadis and Athanassiou, 2017). RCH, a form of phenotypic plasticity, confers survival advantages at low lethal temperature after brief pre-treatment to a prior sub-lethal temperature shock (Lee *et al.*, 1987; Teets and Denlinger, 2013). Over longer time scales such prior exposure to sublethal temperatures also confer advantages to identical future identical thermal stress in what is referred to as beneficial acclimation (Leroi *et al.*, 1994).

In nature, insects may thus face multiple stressors including repeated cold stress during diel and seasonal thermal fluctuations (Marshall and Sinclair, 2010) where the above-mentioned plastic responses play a role (Nyamukondiwa *et al.*, 2018). Mimicking such repeated thermal exposure in manipulative experiments allows investigation of the relationship between repeatability and adaptive responses (Boake, 1989; Morgan *et al.*, 2018; Grinder *et al.*, 2020). In this study, we used common measures of cold tolerance in critical thermal minimum (CT_{min}) and chill coma recovery time (CCRT) as proxies for cold hardiness (Andersen *et al.*, 2015; Mutamiswa *et al.*, 2018, 2019; Izadi *et al.*, 2019).

CT_{min} is an organism's lower thermal tolerance limit where an insect is incapacitated due to compromised neuromuscular activity (Sinclair *et al.*, 2015; Izadi *et al.*, 2019). If low temperature conditions persist, CT_{min} is followed by chill coma where paralysis due to complete loss of neuromuscular function occurs (Hazell and Bale, 2011; O'Neill *et al.*, 2021). The time that an insect requires to regain neuromuscular function following chill coma is what is then regarded as CCRT (Sinclair *et al.*, 2015). Given their ubiquitous occurrence in nature and capacity to define limits for organismal activity, these key indices provide valuable ecologically relevant measures of insect cold tolerance. Thus, understanding the evolutionary capacity following repeated exposure provides important information on their adaptive capacity and potential geographic range expansion in invasive insects such as *Spodoptera frugiperda*.

S. frugiperda is a highly invasive insect pest native to the tropics and sub-tropics of America (Goergen *et al.*, 2016). The larvae of this polyphagous insect cause significant economic losses in several important crops but inflict the most damage in the Poaceae family (Lu and Adang, 1996; Nboyine *et al.*, 2020). In Africa, *S. frugiperda* was first detected in Nigeria before rapidly spreading to 47 countries across the African continent (Goergen *et al.*, 2016; Cock *et al.*, 2017; Early *et al.*, 2018; Nboyine *et al.*, 2020). It is highly destructive to maize, *Zea mays*, which is a staple food in many parts of Africa (Day *et al.*, 2017; Kasoma *et al.*, 2021).

S. frugiperda does not diapause, instead it is known to migrate to environments with favourable conditions for survival (Du Plessis *et al.*, 2020; Vatanparast and Park, 2022). It has been reported to survive in Africa, all year-round due to prevailing

conductive biophysical environment (Early *et al.*, 2018; Du Plessis *et al.*, 2020; Keosentse *et al.*, 2021). The upregulation of glycerol-3-phosphate dehydrogenase and glycerol kinase genes for increased synthesis of the cryoprotectant glycerol has been attributed to the key physiological response to withstand cold environments in *S. frugiperda* (Vatanparast and Park, 2022). However, survival has been reported to be limited in some cases in Asia where harsh winters decimate seasonal populations while annual reinvasions provide new propagules (Vatanparast and Park, 2022). Nevertheless, little is known about the role of acquired/induced cold tolerance in the fitness of *S. frugiperda* following prior exposure. Yet, induced cold tolerance can play a key role in preserving and improving key life-history activities at acute temporal scales.

Here, we examined the consequences of repeated cold exposure on low thermal tolerance (CT_{min} and CCRT) of *S. frugiperda* life stages across 72 h. We hypothesized that CT_{min} and CCRT are repeatable traits and may change over time because of cold hardening. Since body water and lipid content is associated with basal and induced cold tolerance in insects (or lack thereof), we subsequently assessed the two parameters following thermal exposure to draw inferences on the performance of *S. frugiperda* and subsequent management.

Materials and methods

Insect culture and maintenance

The initial colony of *S. frugiperda* was obtained as larvae from the Agricultural Research Council, Plant Health Protection (ARC-PHP) Pretoria, South Africa. Thereafter, the insects were maintained on an artificial diet in the insectary under optimum conditions of 28°C, 65 ± 5% relative humidity (RH) and 12L:12D photoperiod. Since cannibalism is reportedly predominant among late larval instars (Chapman *et al.*, 1999), each third instar larva was individually placed in a separate 100 ml plastic vial with perforated screw-cap lid and soybean wheat germ artificial diet (Southland Products Inc., Lake Village, Arkansas, USA) until pupation. Pupae were maintained in open Petri dishes (30 × 30 × 30 cm³) in collapsible rearing cages made of mesh cloth until adult eclosion. Adults were provided with 25% sugar-water from a moistened cotton wool placed in a Petri dish. At least two maize plants (3–4 weeks old) were placed in each rearing cage as oviposition substrate for gravid females. After hatching, the 1st instar larvae were transferred to an artificial diet for subsequent rearing. For all the experiments F₁ generation of 4th, 5th, 6th instar larvae and 24–48 h old virgin adults were used.

CT_{min} and repeated cold exposure assays

To the relationship between CT_{min} and repeated cold exposure, larvae and adults (males and females) of *S. frugiperda* underwent repeated cold tolerance (CT_{min}) assays at 0 (control), 24, 48 and 72 h intervals. CT_{min} were assayed using standardized dynamic and ecologically relevant protocols (Chidawanyika and Terblanche, 2011; Chidawanyika *et al.*, 2017). Ten replicate larvae and adults were individually placed randomly in a series of 200 mm glass tubes ('organ pipes') connected to an insulated double-jacketed chamber linked to a programmable water bath (Grant model Tx150; Grant Instruments, UK) filled with 1:1 water:propylene glycol. In the 'organ pipes', insects were allowed to equilibrate for 10 min at 28°C (optimum temperature) before

decreasing the temperature at a rate of $0.25^{\circ}\text{C min}^{-1}$ until their CT_{min} were recorded. This was repeated twice for each life stage to yield sample sizes of $n = 20$ per treatment. To record chamber temperature, a thermocouple (type K 36 SWG) connected to a digital thermometer (53/54IIB, Fluke Cooperation, Everett, Washington, USA) was inserted into a control (centre) glass tube of the organ pipes. After each assay, insects were given time to recover before repeating the same assay across 24, 48 and 72 h intervals using the same batch of insects. CT_{min} was considered as the temperature at which insects did not respond to gentle prodding (e.g. Nyamukondiwa and Terblanche 2009).

Influence of repeated cold exposure on CCRT

CCRT was assessed following Mutamiswa *et al.* (2018). A total of ten replicate larvae and adults were placed individually in 7 ml screw-cap glass vials with 1 mm diameter holes pierced through cap for ventilation. The vials were then placed into a large zip-lock bag which was subsequently submerged into a water bath (Grant LTC40 model TX150) filled with a 1:1 water:propylene glycol mixture and set at 0°C for 1 h. After 1 h at chill-coma temperature, the tubes were removed from the water bath and transferred to a Memmert climate chamber (HPP 260, Memmert GmbH+ Co.KG, Schwabach, Germany) set at 28°C , 65% RH for recovery. The chamber was connected to a camera (HD Covert Network Camera, DS-2CD6412FWD-20, Hikvision Digital Technology Co., Ltd, Hangzhou, Zhejiang, China) that was linked to a computer where observations were recorded. This was repeated twice for each life stage to yield sample sizes of $n = 20$ per treatment. After each assay, insects were exposed to the same treatment and CCRT measured across 24, 48 and 72 h intervals using the same batch of insects. CCRT was defined as the time (in min) required for an adult to stand upright on its legs (Milton and Partridge, 2008).

Determination of body water content (BWC)

After 72 h interval following CT_{min} and repeated cold exposure assays, BWC of the insects were determined. Larvae (4th, 5th and 6th instar) and adults were individually placed in a pre-weighed 50 ml Eppendorf tubes and the initial mass of each insect before oven drying was measured (to 0.0001 g) on a Scout Pro (DHAUS) microbalance (model: Scout Pro SPU 123, Parsippany, USA). Thereafter, insects were placed in a Memmert drying oven (UL50, Memmert, Schwabach, Germany) set at 60°C for 72 h. Insects were allowed to cool under laboratory temperature conditions of 28°C for 30 min thereafter, dry mass was measured (to 0.0001 g) on a microbalance. To determine BWC, dry mass was subtracted from the initial mass following Bazinet *et al.* (2010) and Weldon *et al.* (2018).

Determination of body lipid content (BLC)

Following BWC assays, the tested insects were further oven dried for another 72 h at 60°C . Thereafter, the insects were individually washed in 1.5 ml diethyl ether and then gently agitated at 250 rpm for 24 h at 37°C using ST 5 CAT orbital shaker (model: Zipperer GmbH, D 79219 Staufen, Germany) following the methods of Mitchell *et al.*, (2017). The diethyl ether was then removed from the tubes and insects were oven dried again at 60°C for 24 h, before reweighing. The lipid content for each individual was calculated by subtracting the lipid-free dry mass from the

initial dry mass. Controls were exposed to the same conditions before measuring their lipid content.

Data analysis

Data analyses were carried out in STATISTICA, 13.5.0 version (Statsoft Inc., 2021) and R version 4.1.2 (R Development Core Team, 2021). Normality and equality of variances were first checked using the Shapiro–Wilk and Hartley–Bartlett tests, respectively. Data for CCRT was linear and met the conditions for normality and equality of variances ($W = 0.83$, $P = 0.12$) and were analysed using generalized linear models assuming a Gaussian distribution and an identity link function in R. The CT_{min} data also met the linear model assumptions and were analysed using repeated measures analysis of variance. Tukey–Kramer's *post-hoc* tests were used to separate statistically heterogeneous means. The relationship between CT_{min} and BWC and BLC were examined using linear regression in STATISTICA.

Results

CT_{min} and repeated cold exposure assays

CT_{min} significantly varied across life stages following repeated cold exposure ($F_{16, 282} = 134.59$, $P < 0.001$) (fig. 1). In 5th instar and virgin adults, cold tolerance (CT_{min}) improved with repeated cold exposure (fig. 1). However, 6th instar larvae showed compromised cold tolerance with CT_{min} increasing with repeated exposure (fig. 1). Virgin females recorded the lowest CT_{min} across all assays relative to other life stages (fig. 1).

CCRT and repeated cold exposure assays

As in CT_{min} assays, CCRT varied significantly across life stages with repeated cold exposure ($F_{16, 282} = 4.06$, $P < 0.001$) (fig. 2). CCRTs of tested instars (4th, 5th and 6th instar) decreased with repeated cold exposure (fig. 2). In adults (virgin males and females), CCRT improved following repeated exposure at 24 h interval and was compromised after 48 and 72 h intervals (fig. 2).

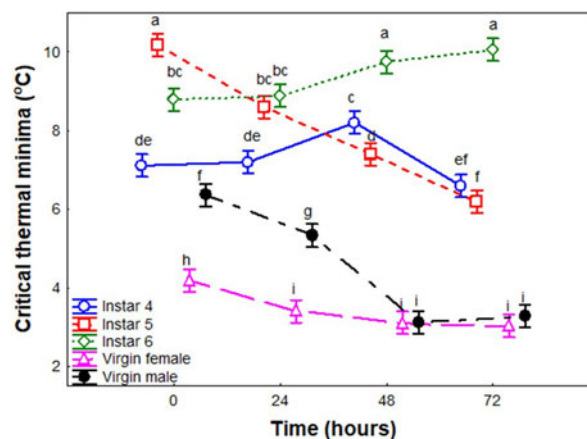


Figure 1. CT_{min} in adult (virgin male and female) and larval stages of *S. frugiperda* following repeated cold exposure. Data points represent means of $n = 20$ while error bars denote 95% confidence limits for each gender and life stage. Different letters above error bars denote significant differences.

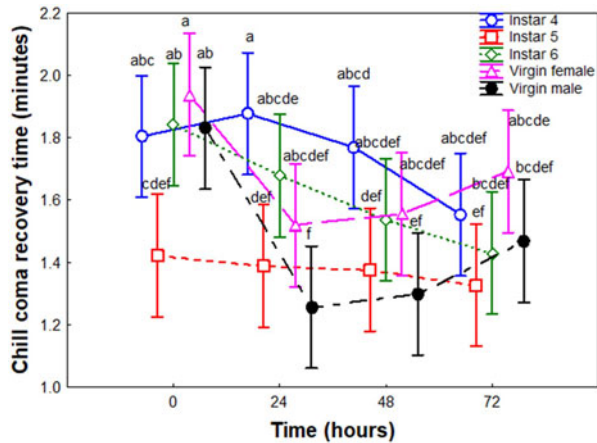


Figure 2. CCRT in adult (virgin male and female) and larval stages of *S. frugiperda* following repeated cold exposure. Data points represent means of $n=20$ while error bars denote 95% confidence limits for each gender and life stage. Different letters above error bars denote significant differences.

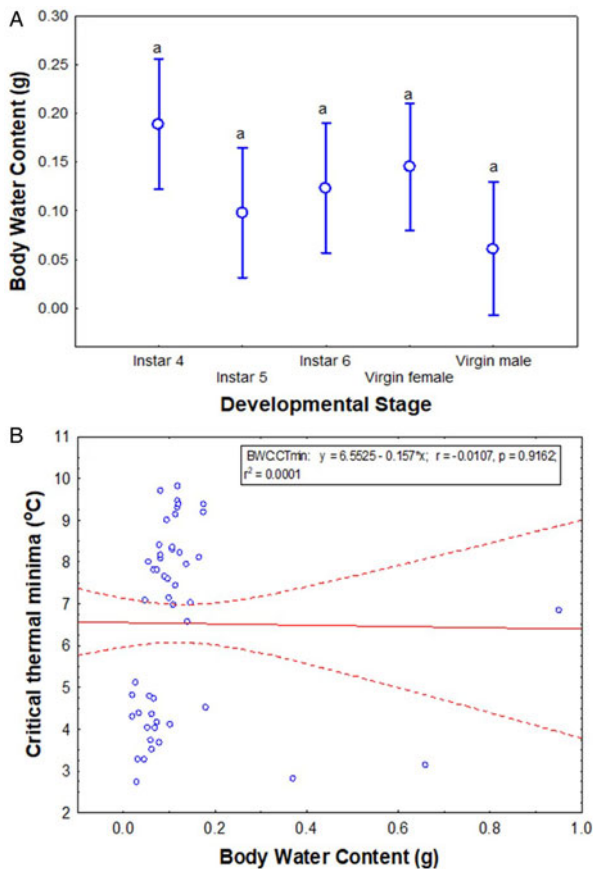


Figure 3. BWC (g) across different life stages (A) and relationship between BWC and CT_{min} (B) in *S. frugiperda*.

Body water and lipid content

BWC did not vary significantly among life stages ($F_{4, 95} = 2.01$, $P = 0.98$) (fig. 3A). There was no significant difference in BWC between all tested life stages (fig. 3A). Nevertheless, BWC was not significantly correlated with low temperature tolerance (measured as CT_{min}) (fig. 3B).

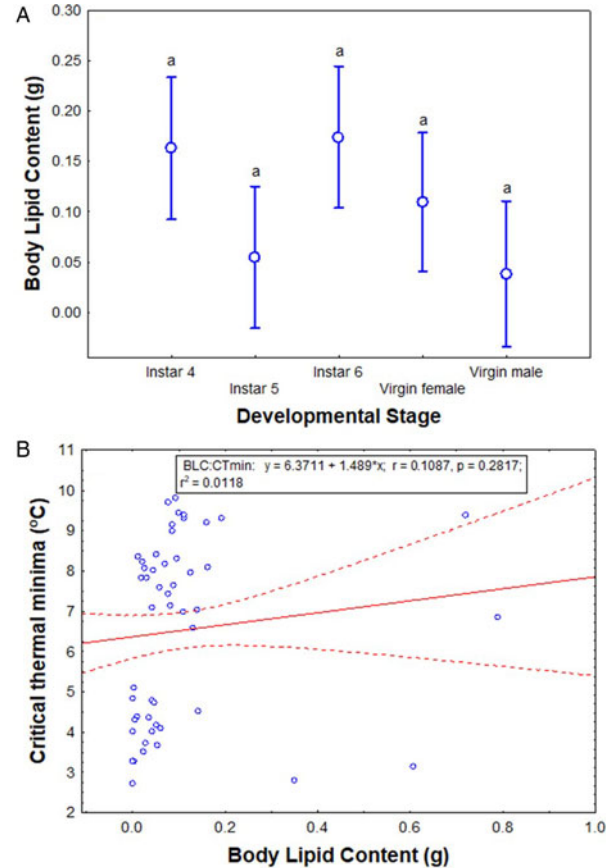


Figure 4. BLC (g) across different life stages (A) and the relationship between BLC and CT_{min} (B) in *S. frugiperda*.

Similar to BWC, BLC did not significantly vary among life stages ($F_{4, 95} = 2.94$, $P = 0.24$) (fig. 4A). As in BWC, BLC was not significantly correlated with low temperature tolerance such that CT_{min} decreased with BLC (fig. 4B).

Discussion

Insect physiological and behavioural adaptations are very important for determining survival and population dynamics in both transient and seasonal cold spells (Chown and Nicolson, 2004; Terblanche *et al.*, 2011; Andrew and Kemp, 2016). As expected, our results showed that repeated cold exposure influences the fitness of *S. frugiperda* (determined as CT_{min} and CCRT). While insects may face multiple temperature variabilities in winter season, the repeated cold exposures can trigger responses that may set the insect on a different physiological path relative to a single exposure (Marshall and Sinclair, 2010, 2012). In the current study, CT_{min} improved with repeated exposure in 5th instar larvae, virgin males and females in agreement with Renault *et al.* (2004) who reported improved survival in beetles that were exposed to repeated cold exposure. A similar trend was reported in *Drosophila melanogaster*, with low temperature tolerance improving following repeated cold exposure in tested insects (Le Bourg, 2007). However, compromised and fluctuating CT_{min} were recorded in 6th instar and 4th instar larvae, respectively. Given this variation across life stages, it therefore indicates that repeated thermal exposure impacts on CT_{min} are life-stage dependent.

While 5th instar larvae, virgin males and females showed enhanced CT_{min} across subsequent exposures, virgin females recorded the lowest CT_{min} across treatment intervals indicating that they were the most thermally tolerant. This gives them a fitness and survival advantage when they encounter extreme cold conditions in nature.

In the present study, repeated thermal exposure improved CCRT in 4th, 5th and 6th instar larvae and this is in consonance with Andersen *et al.* (2017) who reported improved chill-coma recovery, cellular survival and cold tolerance in *Locusta migratoria* following brief cold exposure periods. However, compromised CCRTs were recorded in adults (males and females) in keeping with van Dooremalen *et al.* (2011) who reported CCRT decrease in *Orchesella cincta* following repeated cold exposure. The variations in the current study underlie that CCRT responses are life-stage dependent. Although CCRT and CT_{min} are measures of cold tolerance, surprisingly, 6th instar larvae recorded compromised CT_{min} and enhanced CCRT indicating that responses also vary across traits, thus can be trait dependent.

The changes in cold tolerance across consecutive measurements provide insight into potential benefits of short-term acclimation to extreme cold events through cold hardening. Our results showed evidence of cold hardening in *S. frugiperda* as indicated by improved cold tolerance in some of the life stages. This suggests significant adaptive potential for cold tolerance in this invasive insect species and that individuals may also respond directly to low temperature extremes through phenotypic plasticity. While *S. frugiperda* has been reported to overwinter and survive all year round in Africa (Kebede and Shimalis, 2018; Prasanna *et al.*, 2018; Keosentse *et al.*, 2021), the results indicate its potential to adapt to variable thermal extremes in winter and this may give it fitness and survival advantage in the face of climate change. Insects reportedly enhance their cold tolerance through carbohydrate cryoprotectants accumulation, antifreezes synthesis, lipid membranes reordering and either removal (freeze avoiding) or retaining (freeze tolerant) of ice nucleators (Lee, 2010). Therefore, differential life-stage responses shown in this study following repeated exposure assays may be a result of variation in these physiological components of cold hardiness. However, this warrants further investigation to fully elucidate the responses.

Cold tolerance is dependent on the water content remaining unfrozen in many cold hardened insects by allowing basal metabolism to continue at low temperature levels (Colinet *et al.*, 2007; Alfaro-Tapia *et al.*, 2021). Reports have shown that reduction in BWC and subsequent increase in solute concentration may increase cold tolerance in insects (Worland, 1996). In the current study there was no relationship between cold tolerance and BWC. This may be because insects in our assays did not experience repeated cold conditions that trigger any water loss and subsequent solute concentration increase. While Keosentse *et al.* (2021) reported that BWC increased with larval stage in *S. frugiperda*, our results report otherwise on CT_{min} following repeated exposure. This may be because our present study measured BWC following plastic responses while Keosentse *et al.* (2021) measured basal BWC. Given these responses, it indicates that *S. frugiperda* may trade-off basal BWC for plasticity of thermal tolerance.

Lipid content plays a vital role in cold tolerance as they can serve as anti-freezers in the insect haemolymph (Sinclair and Marshall, 2018; Trenti *et al.*, 2022). In winter, most insects do not feed and may face the unreplaced energy consumption, water loss and low temperatures (Sinclair *et al.*, 2013; Williams *et al.*, 2015). Low temperature is one of the stressors which affect

neutral lipid fluidity and mobilization and energy drain, since lipids are the primary overwintering source of fuel (Sinclair and Marshall, 2018). As such, most overwintering insects end winter with fewer lipid stores than at the beginning (Sinclair, 2015). For example, in laboratory-reared colonies of *D. melanogaster*, glycogen levels decreased following repeated cold exposure (Marshall and Sinclair, 2010). In addition, there was a positive correlation between BLC and cold tolerance in *Drosophila* spp. (Hoffmann *et al.*, 2001; Kaczmarek and Boguś, 2021). However, in the current study, our results showed no significant correlation between BLC and cold tolerance in *S. frugiperda*. A recent study attributed glycerol as the key cryoprotectant used by *S. frugiperda* (Vatanparast and Park, 2022). This therefore suggests that the influence of BLC on cold tolerance may be species dependent and glycerol maybe more important in this species.

In conclusion, the current study documents life-stage-related variation in cold tolerance for *S. frugiperda* following repeated thermal exposure. Our results suggest that repeated cold exposure differentially influences the fitness of *S. frugiperda* in nature where vulnerability is life-stage and trait dependent. In addition, the study provides evidence that cold hardening may be an important mechanism for *S. frugiperda* to cope with repeated cold exposure over the short term. These cold tolerance responses may provide temporal fitness benefits following repeated cold conditions in nature hence population persistence under changing environments. The results also have direct implications on the geographic distribution of the pest under climate change scenarios where warming winter seasons will lead to even further spatial expansion and multivoltinism due to favourable conditions. For a polyphagous pest such as *S. frugiperda* this will be critical as alternative hosts will support multiple generations enough to exert pest pressure on the main crop in the subsequent season (Vatanparast and Park, 2022). In such cases, management practices should consider area-wide monitoring of the pest populations even during off-season for early integrated pest management practices. This may include improved phytosanitary measures and reduction of alternative hosts on-farm. More importantly, augmentative releases to boost parasitoid populations during this period will also be a feasible option to suppress the pest populations to reduce the pressure in the main crop in the impending season. This will greatly reduce pest pressure, but costs are associated with control of the outbreak pest using synthetic pesticides on-season. Future studies should therefore determine the intensity of such parasitoid levels to maintain pest pressure well below economic injury levels.

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