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Effect of different lethal temperature assay methods on thermal tolerance plasticity of three different breeds of mulberry silkworm (*Bombyx mori* L.)

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Abstract

Background: Mulberry silkworm (*Bombyx mori* L.) is one of the best-studied insect models, regularly used as a type specimen for thermal tolerance experiments on insects. Still, the upper lethal limit of this lepidopteran has never been explored extensively using any sort of conventional lethal assay method. The present study deals with the employment of different lethal assay protocols for the study of survivorship of three different breeds of mulberry silkworm (*B. mori*) exposed to different temperatures (30–50 °C) and durations of stress (1–3 h) on different days (day 2, 4, and 6) of the fifth instar stage for formulating an extensive upper lethal temperature (ULT_{50} and ULT_{25}) index.

Results: Among treatment temperatures 30 °C, 35 °C, and 40 °C had a significant ($p = < 0.0001$) impact on the high-temperature survival rate of the silkworm. Among duration—1 h and 2 h influenced the survival rate significantly ($p = < 0.0001$). Plunging, one-way ramping, and two-way ramping assay methods seemed to exert a non-significant (Wald $\chi^2 = 3.253$, $p = 0.197$) influence on silkworm survival. F1 hybrid was found to exhibit the highest survivorship across different temperatures, followed by the multivoltine Nistari plain and then by the bivoltine breeds. In F1 hybrid silkworms, the upper lethal temperatures ULT_{50}, varied within the range of 37 °C to 44 °C and ULT_{25} varied within the range of 40–47 °C. The mean upper lethal limit—ULT_0 for all three breeds of mulberry silkworm, across all experimental groups, was computed to be ~ 49 °C.

Conclusions: Ultimately the overall thermal tolerance of mulberry silkworm exhibited a significant inter-breed variation based on the heterogeneous thermal plasticity of the three different breeds. The outcome of the present study in the form of upper lethal temperature ranges of the breeds under consideration can form the basis of future thermal stress experiments in mulberry silkworms.

Keywords: Thermal tolerance plasticity, Upper lethal temperature, Lethal temperature assay, Plunging, Ramping

Background

Ambient climatic conditions dictate vital aspects of physiology and developments in most of the poikilotherms, and insects are no exception. Domination of temperature, among all the environmental variables, is probably most pronounced for insect life stages (Chidawanyika & Terblanche, 2011). The thermal tolerance plasticity of an insect shapes the geographical distribution and abundance of the species to a great extent. On the other hand, species distribution can significantly influence physiological tolerance limits. A combination of factors like these makes it difficult to establish a 'universal' thermal limit (Overgaard et al., 2012) in insects.

The effect of an experimental setup such as plunging (constant target temperature) or ramping (incremental

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target temperature) assay methods on insect thermal tolerance limits is evident from various recent studies (Bahar et al., 2012; Mitchell & Hoffmann, 2010; Nguyen et al., 2014; Rezende et al., 2011; Terblanche et al., 2011). Most of the physiological adjustment mechanisms are not instantaneous and require suitable exposure time. Therefore, 'ramping assays' involving gradual rise from ambient to target stress temperature are considered to be more relevant and akin to natural conditions. Whereas, the plunging assays, involving a sudden dip or hike to the target temperature can become more stressful for the insect specimens (Nguyen et al., 2014; Overgaard et al., 2012). Therefore, when it comes to the evaluation of thermal response, it is more weighted towards ramping assay methods with incremental temperature change than towards constant temperature direct plunging assay. However, for high-temperature assays, no significant difference in survivability was observed between plunging and two-way ramping assays (Nguyen et al., 2014). Among the ramping assay methods, however, the one-way ramping, involving a gradual rise in assay temperature to the desired stress level and then a sudden return to the ambient or basal temperature, can itself be a 'thermal shock' (Nguyen et al., 2014), whereas the two-way ramping technique as advised by Nguyen et al. (2014) in the case of Diamondback moth (DBM) *Plutella xylostella* and Sinclair et al. (2004) in the case of sub-Antarctic caterpillar *Pringleophaga marioni* deals with a gradual decrease in temperature to the basal temperature threshold after the shock period is over.

The fact that temperate populations are less susceptible to cold stress but not to heat stress (Petavy et al., 2004) further advocates that silkworms, being temperate lepidopterans, find it hard to adapt to tropical tyranny. The mulberry silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), is one of the best-studied insect genetic models (Joy & Gopinathan, 1995; Kimura et al., 2001; Rahmathulla, 2012; Tazima, 1978), second only to the fruit fly, *Drosophila melanogaster* (Nagaraju, 2000). It is also one of the most reared economic insects worldwide (Meng et al., 2017; Soumya et al., 2017). Naturally, it has been a target of much research. Stress response in silkworms has been an area of active focus in modern biology. But the majority of experimental studies to date, dealing with thermal tolerance plasticity of silkworms, relied heavily on a constant temperature laboratory setup (Greiss & Petkov, 2001; Kumar et al., 2001; Kumari et al., 2001). Moreover, only a very few of them involved the assessment of lethal temperatures following a well-structured and conventional thermal lethality assay and hence were not suitable enough for formulating a universal thermal limit index (Overgaard et al., 2012). The determination of the sub-lethal threshold of the stress forms one of the primary prerequisites of any thermal stress response experiment and depends upon the evaluation of the lethal temperature limit. Therefore, higher extreme temperature experiments involve assessment of lethal temperature limit or ULT_0: marked by 0% survival of the experimental population and upper lethal temperature (ULT) points, viz. ULT_{50} and ULT_{25}, at which, respectively, 50% and 25% of the population survive. These thermal estimates are the most fundamental of the physiological responses related to stress, and their assessment is rapidly gaining much emphasis in research concerning stress response and the effect of climate changes on species distribution patterns (Andrew, 2013; Andrew et al., 2013a, 2013b; Angilletta, 2009). When it comes to the silkworm stress response study, any of such lethal temperature points are yet to be established.

Methods

Laboratory conditions

The work was conducted during April to August (extended summer months) for three consecutive years up to 2019, at the Sericulture Research Laboratory, Post Graduate Department of Zoology, Hooghly Mohsin College, Chinsurah, Hooghly, West Bengal, India (22° 52′ 58.077″ N, 88° 24′ 1.845″ E), to evaluate the upper thermal limit during the Indian summer months. Climate data, viz. maximum, minimum, and average daily temperature and relative humidity, were recorded at the laboratory using a dry–wet bulb thermometer throughout this period.

The specimen

Mulberry silkworm—*Bombyx mori* L. (Lepidoptera-Bombycidae), was used for this study. Eggs of disease-free layings of three locally available breeds of mulberry silkworm, viz. the multivoltine breed—Nistari plain (NP), one bivoltine (BV) breed (SK6 X SK7 hybrid), and one F1 hybrid breed (from a cross between Nistari plain and SK6 X SK7 hybrid), were procured as Egg-cards from Ranaghat and Shibnibas extension farms of State Sericulture Directorate, West Bengal, India, and reared in the rearing room under natural environmental conditions. Larvae were fed with fresh mulberry leaves of improved variety-S_{1635}, procured from the mulberry plantation, Hooghly Mohsin College. Then, 2-, 4-, and 6-day-old silkworm larvae of the fifth instar stage were used for thermal tolerance experiments. After more than five thousand years of domestication, mulberry silkworm larvae do not need further acclimatization in an anthropogenic environment.

Experimental setup

Fifth instar larvae of mixed gender (individuals were chosen randomly to generalize result, irrespective of sex) were divided into three groups—viz. D1 (day 2), D2 (day 4), and D3 (day 6). One hundred individuals from each group were then exposed to four temperature ranges (set by conducting trials to estimate 100% to 0% survivability, before treatments)—viz. T1 (35 °C), T2 (40 °C), T3 (45 °C), and T4 (50 °C), for three different durations of stress—viz. S1 (1 h), S2 (2 h), and S3 (3 h). A control set (T0) was maintained at room temperature (~ 30 °C). All the experiments were done in triplicates. Experiments with the setup mentioned above were repeated using plunging (P), one-way ramping (R1), and two-way ramping (R2) methods (Fig. 1) following Nguyen et al. (2014). A brief description of the methods is stated below.

Plunging assay

The total number of individuals from each experimental group was divided into smaller groups with an equal number of individuals and put into non-airtight plastic vials (fitted with a thermocouple which was connected to a data logger). Vials were then plunged into the programmable water bath maintained at the desired temperature and kept there for stipulated durations. Upon successful completion of the treatment period, all the vials were removed from the water bath, and the larvae were kept at room temperature (~ 30 °C) for 24 h before the number of surviving individuals was recorded for each experimental group (Fig. 1).

One-way ramping assay

Instead of dipping vials directly into the target temperature like the plunging assay, the temperature of the water bath was maintained at 30 °C for the first 10 min and then was gradually increased to the target temperature. Larvae were kept at the target temperature for desired exposure time, and after that, vials were removed.

Two-way ramping assay

Similar to the one-way ramping method, the temperature of the water bath was slowly raised to meet the desired temperature ranges, but unlike the previous two methods, the larvae were still kept in the water bath even after the exposure duration was over, and the temperature was gradually decreased to 30 °C (T0).

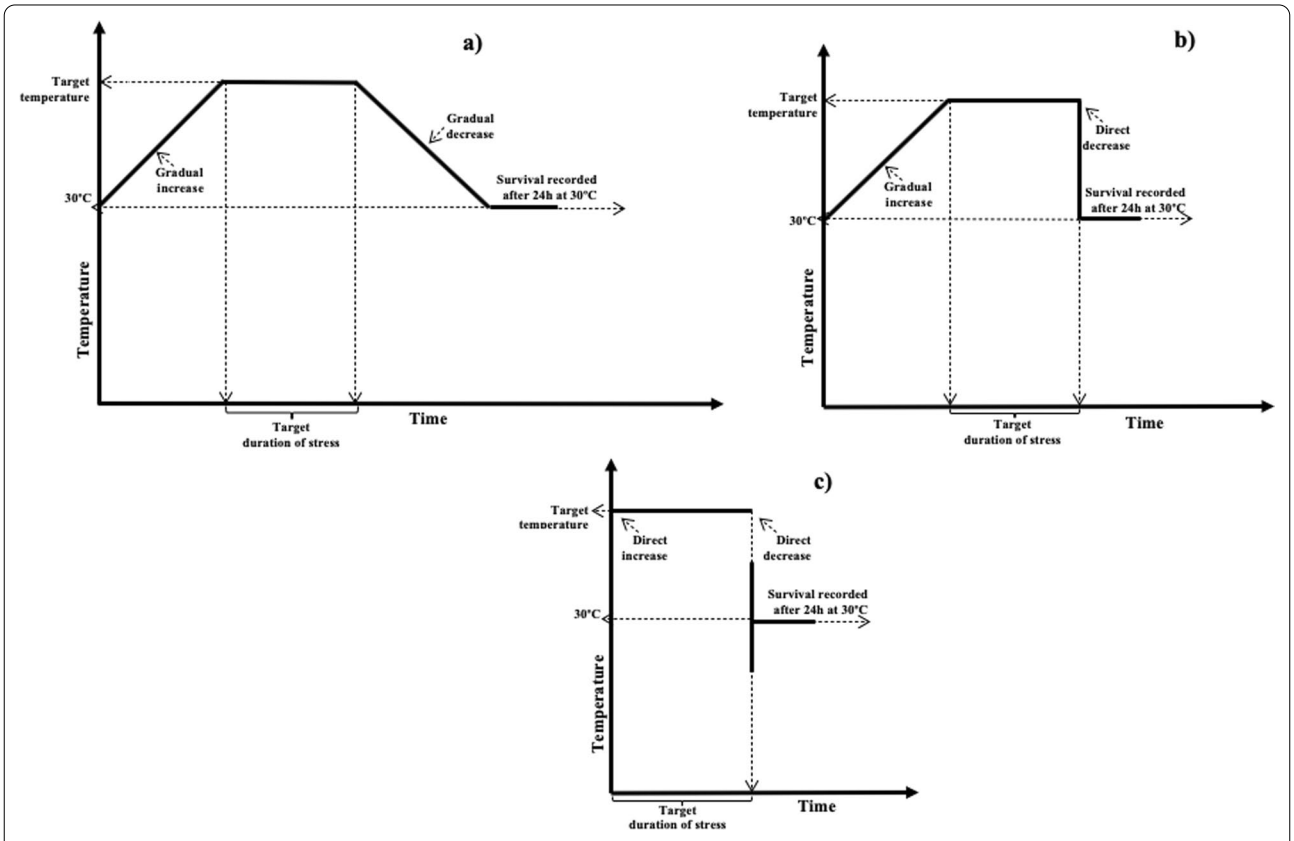

Fig. 1 Schematic representation of different lethal assay methods: **a** two-way ramping, **b** one-way ramping, **c** direct plunging

Statistical analyses

Estimation of upper lethal temperatures (ULT_{50} and ULT_{25})

The proportion of surviving individuals recorded from each experimental set was used to compute the upper lethal limit (ULT_0) and the upper lethal temperatures (ULT_{50} and ULT_{25}). Recorded data were analysed, following the standard method (Andrew et al., 2011, 2013a; Nguyen et al., 2014; Terblanche et al., 2008) using probit regression in the IBM SPSS statistics (version 26 for MAC OSX).

Comparison of experimental treatment groups

Survival rate and upper lethal temperature data obtained from each experimental treatment group were compared using the following statistical methods. ANOVA, followed by Tukey–Kramer post hoc tests (Assaad et al., 2015), was done to illustrate the effect of different temperature ranges and durations of exposure on the ULT data, whereas generalized linear model (GLM) type III was performed in IBM SPSS statistics (version 26 for MAC OSX) to analyse the effect of different assay methods, temperature ranges, durations, and breeds on survivability of the silkworm larvae. As survival was recorded as a proportion of dead or alive, therefore the custom model was run following a slight modification of the method adopted by Terblanche et al. (2008) assuming a normal distribution and log link function using interaction as build term, hybrid parameter estimation method with scale parameter method set to fixed value -1, and the criteria method set as—Fisher (1).

Results

Climate data

The mean daily temperature recorded at the laboratory, from April to August (extended summer months) for three consecutive years up to 2019, was found to be within the range of 30 ± 1 °C. Based on this observation, the basal point of thermal stress was set at 30 °C (T0). Recorded temperature data during this period are plotted in a line graph (Fig. 7-supplementary information) and presented in Additional file 1.

Survival rate

The overall survivorship of the mulberry silkworm reared during the extended Indian summer months varied accordingly to the temperature and duration of stress (Figs. 5, 6). Moreover, stress applied on different days of the fifth instar stage seemed to have varying influences on the survivability of mulberry silkworm as well (Table 1, Fig. 3: Red markers indicate significant differences, $p < 0.05$). The survival data reflected a significant impact upon changes in temperature and duration of exposure to the treatment sets (Table 2, Figs. 5, 6). The result from the GLM analysis pointed out temperature as the most dominant predictor variable among the factors (Table 1, Fig. 5). Apart from temperature, the duration of stress was also observed to be another significant influencing factor (Table 1, Fig. 6). Different assay methods adopted for each experiment seemed to exert a non-significant (Wald $\chi^2 = 3.253$, $p = 0.197$) influence on silkworm survival. Therefore, owing to the maximum survivorship, unless otherwise mentioned, all further statistical analysis (Table S6) and visualization of corresponding data (Figs. 2, 3, and 4) were based on survival and upper lethal limit data obtained from the two-way ramping experiments. Among the different temperature ranges used in the experiments, T0, T1, and T2 had a significant ($p = < 0.0001$) impact on the high-temperature survival rate of the silkworm. Among these three temperature

Table 1 Comparison of effects of different predictor variables, viz. temperature, duration of stress, day, assay, breed, and their interaction through a generalized linear model of regression on the survivorship of summer reared mulberry silkworm (*Bombyx mori* L.)

Parameter	Type III		
	Wald χ^2	*df*	*p*
(Intercept)	44,322.019	1	**< 0.0001**
Temperature	1190.597	3	**< 0.0001**
Duration of stress	94.330	2	**< 0.0001**
Day	21.729	2	**< 0.0001**
Assay	3.253	2	0.197
Breed	53.880	2	**< 0.0001**

Significant differences are marked in bold

Table 2 Survivorship of mulberry silkworm (*Bombyx mori* L.) analysed using a generalized linear model of regression, showing variation depending on different temperatures and durations of stress

Parameter	Wald χ^2	*df*	*p*
(Intercept)	26,048.875	1	**< 0.0001**
T0	53,768.624	1	**< 0.0001**
T1	50,978.296	1	**< 0.0001**
T2	45,902.724	1	**< 0.0001**
T3	Na	Na	Na
T4	Na	Na	Na
S1	92.574	1	**< 0.0001**
S2	20.182	1	**< 0.0001**
S3	Na	Na	Na

Significant differences are marked in bold. Na indicates no analysis was carried out either because of almost no survival across all treatments or maybe due to the last unique category in each predictor variable which was associated with redundant parameter in the estimation algorithm

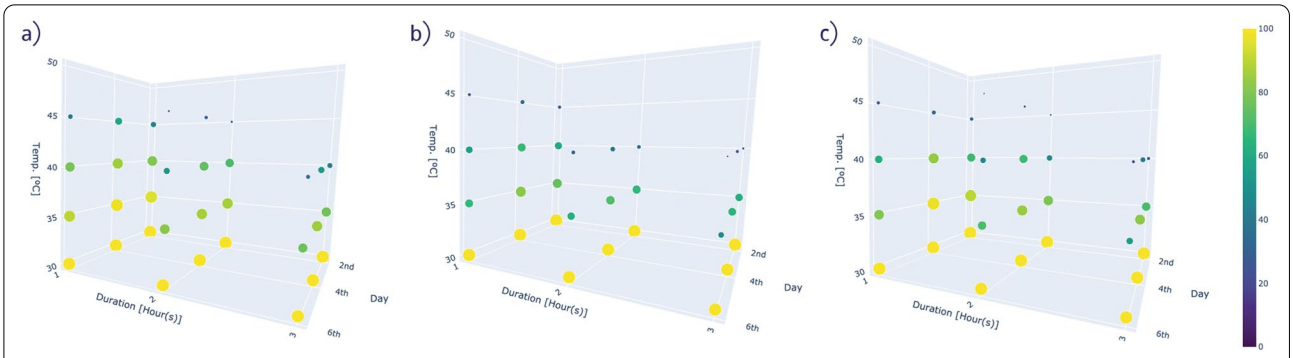

Fig. 2 Survivorship of mulberry silkworm (*Bombyx mori* L.) following two-way ramping assay protocol, across different experimental groups visualized using a 3D scatterplot. The colour and size of the markers were linked to survival %, and only nonzero values are visible; **a** F1 hybrid, **b** bivoltine breed, **c** Nistari plain breed

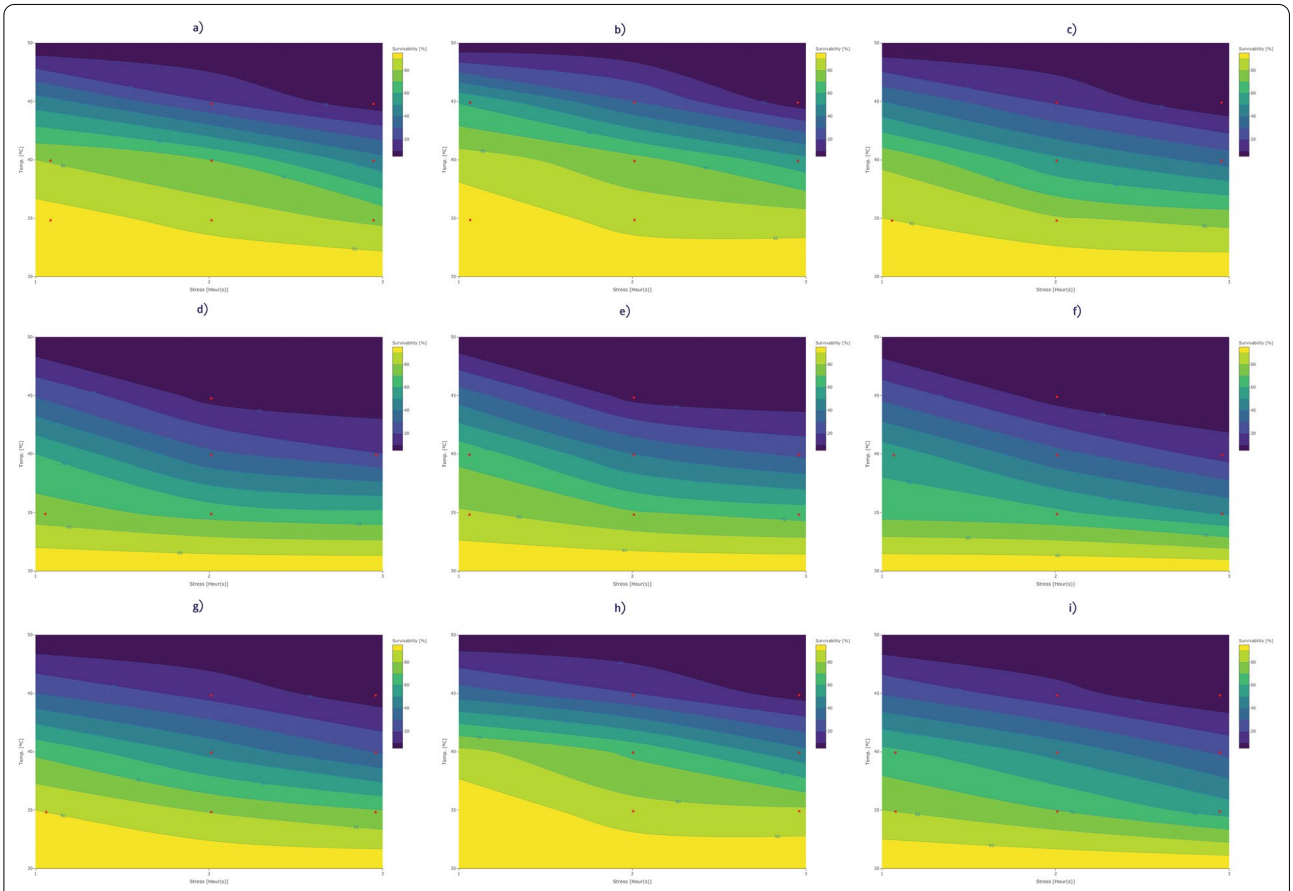

Fig. 3 Contour plot showing comparisons of silkworm survivorship (deep purple—0% to yellow—100% survival) in the two-way ramping assay, across different breeds, for different temperatures and duration of stress, depending on diurnal variation in high-temperature tolerance. Red markers indicate significant differences ($p < 0.05$) in survival across different experimental groups. F1 hybrid D1–D3 (**a**–**c**), bivoltine D1–D3 (**d**–**f**), and Nistari plain D1–D3 (**g**–**i**)

ranges, T0 (30 °C) accounted for the maximum Wald χ^2 value, as survival was maximum at room temperature across all the experimental groups. However, T1 (35 °C) and T2 (40 °C) also seemed to impart a substantial impact on survival, as was evident from the respective Wald χ^2 values, whereas T3 (45 °C) and T4 (50 °C) seemed to account for negligible variation in survivorship (Table 2). A comparison of the effect of different durations of stress

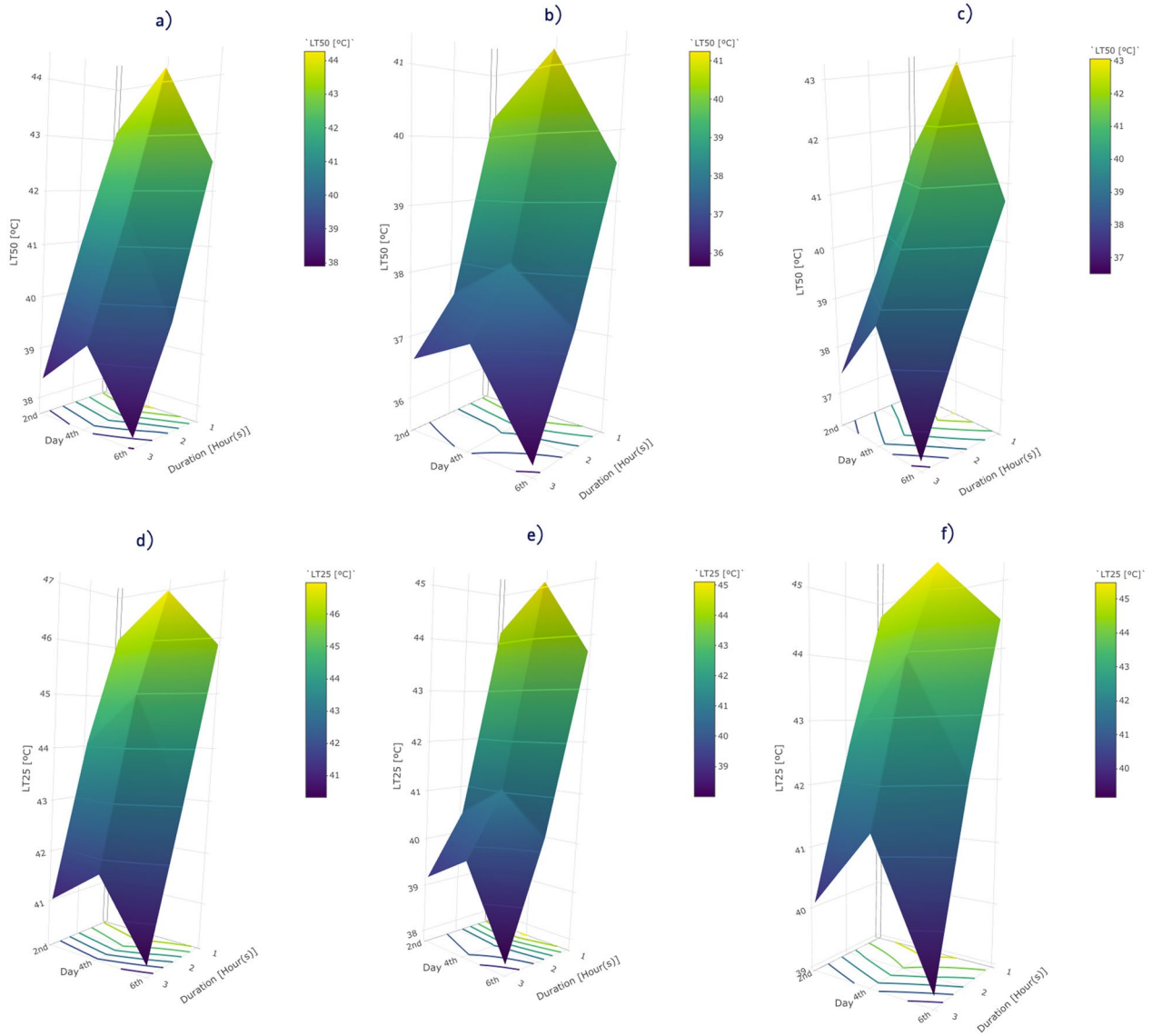

Fig. 4 Plasticity of upper lethal limits (ULT_{50} and ULT_{25}) of mulberry silkworm (*Bombyx mori* L.) in the two-way ramping assay, across different breeds demonstrated by 3D surface plots combined with 2D contour plots. **a** F1 ULT_{50}, **b** bivoltine ULT_{50}, **c** Nistari plain ULT_{50}, **d** F1 ULT_{25}, **e** Bivoltine ULT_{25}, **f** Nistari plain ULT_{25}

on silkworm survival revealed that both S1 (1 h) and S2 (2 h) had a significant ($p = < 0.0001$) influence on the survival rate. However, exposing larvae for an hour to the target temperature seemed to have a more significant impact (Wald $\chi^2 = 92.574$) than that for 2 h (Wald $\chi^2 = 20.182$) on the proportion of surviving individuals.

Lethal limits

The high-temperature lethal spectrum computed from the silkworm survival data obtained from thermal tolerance experiments, using different temperature ranges, and different durations of stress for three different breeds of mulberry silkworms treated on different days of the fifth instar stage, during the extended Indian summer months (April–August), for three consecutive years up to 2019 are presented in Table 3. The mean upper lethal limit—ULT_0 (the temperature at which 0% individuals of the experimental population survived) for all three breeds of mulberry silkworm, across all experimental groups was computed to be ~ 49 °C, Whereas the variation in upper lethal temperatures ULT_{50} and ULT_{25} for *Bombyx mori* L. was computed to

Table 3 Upper lethal temperature spectrum of three different breeds of mulberry silkworm (*Bombyx mori* L.) at different temperatures and durations following different assay methods

Assay	Treatment	F1		BV		NP	
		ULT50	ULT25	ULT50	ULT25	ULT50	ULT25
P	S1D1	42.369	45.769	39.566	43.464	40.796	44.152
	S2D1	40.177	43.366	36.804	39.359	38.594	41.729
	S3D1	37.673	40.215	35.937	38.1	36.726	39.15
	S1D2	43.421	46.89	40.443	44.407	42.145	45.119
	S2D2	40.955	44.391	37.495	40.098	40.088	43.274
	S3D2	38.534	40.931	36.396	38.677	38.006	40.472
	S1D3	41.856	45.347	38.921	42.921	40.077	43.74
	S2D3	38.926	42.122	36.517	39.015	37.899	41.074
	S3D3	37.373	39.732	35.157	37.179	36.012	38.471
R1	S1D1	42.788	45.955	39.958	43.843	41.203	44.455
	S2D1	40.521	43.695	37.131	39.875	38.926	42.122
	S3D1	37.964	40.541	36.221	38.538	37.013	39.523
	S1D2	43.826	47.08	40.837	44.822	42.562	45.399
	S2D2	41.249	44.721	37.783	40.6	40.378	43.633
	S3D2	38.881	41.372	36.729	39.256	38.346	40.93
	S1D3	42.155	45.643	39.205	43.32	40.367	44.101
	S2D3	39.406	42.66	36.983	39.776	38.367	41.674
	S3D3	37.605	39.982	35.384	37.54	36.236	38.767
R2	S1D1	43.155	46.16	40.302	44.228	41.558	44.768
	S2D1	40.929	44.054	37.512	40.435	39.318	42.562
	S3D1	38.406	41.047	36.649	39.186	37.445	40.092
	S1D2	44.267	46.978	41.245	45.103	43.051	45.474
	S2D2	41.599	45.002	38.112	41.044	40.723	43.952
	S3D2	39.275	41.743	37.101	39.749	38.734	41.329
	S1D3	42.509	45.871	39.543	43.698	40.71	44.428
	S2D3	39.744	43.018	37.306	40.257	38.695	42.074
	S3D3	37.9	40.32	35.662	38.003	36.515	39.154

P, plunging assay; R1, one-way ramping assay; and R2, two-way ramping assay

Table 4 Plasticity of upper lethal limits of mulberry silkworm (*Bombyx mori* L.) showing inter-breed variation

ULT_{50}	BV	F1	NP
	37.8 ± 0.34^{b}	40.5 ± 0.407^{a}	39.3 ± 0.38^{a}
ULT_{25}	40.8 ± 0.474^{b}	43.5 ± 0.466^{a}	42.3 ± 0.422^{ab}

Values are means ± SEM, $n = 27$ per treatment group. Means in a row without a common superscript letter differ ($p < 0.05$) as analysed by one-way ANOVA and the Tukey test

be within the range of 35–44 °C and 37–47 °C, respectively. The lethal limits exhibited significant variation across the experimental groups (Table 4), but no significant deviation in the upper lethal temperatures was observed across different assay methods (Table 5) for all three breeds of the mulberry silkworm.

Thermal plasticity of the mulberry silkworm

Plasticity of survival

Thermal plasticity in mulberry silkworm as reflected from the survival rate of three different breeds of *Bombyx mori* L. when exposed to the varying magnitude of thermal stress seemed to exhibit an inter-breed variation (Figs. 2, 5, 6). The basis of this statement stems from the fact that 'Breed' as a predictor variable exerted a significant ($p < 0.0001$) influence on the overall rate of survival across different treatment groups. The effect of 'Breed' seemed to be more significant in comparison with that of 'Day' (Table 1). Therefore, the proportion of surviving individuals after each treatment varied significantly across different breeds. The shift in overall survivorship depicted in Fig. 5 illustrated the F1 hybrid having the highest survivorship across different temperatures, followed by the multivoltine Nistari plain and then by the bivoltine breeds. The effect of different durations on the

Table 5 Effect of different assay protocols on upper lethal temperatures of silkworm breeds

Breed	Upper lethal temperatures	One-way ramping	Plunging	Two-way ramping
F1	ULT50	40.5 ± 0.729	40.1 ± 0.718	40.9 ± 0.733
	ULT25	43.5 ± 0.844	43.2 ± 0.862	43.8 ± 0.799
BV	ULT50	37.8 ± 0.606	37.5 ± 0.596	38.2 ± 0.61
	ULT25	40.8 ± 0.849	40.4 ± 0.862	41.3 ± 0.82
NP	ULT50	39.3 ± 0.678	38.9 ± 0.665	39.6 ± 0.688
	ULT25	42.3 ± 0.762	41.9 ± 0.771	42.6 ± 0.728

Values are means ± SEM, $n = 27$ per treatment group. Means in a row without a common superscript letter differ ($p < 0.05$) as analysed by one-way ANOVA and the Tukey test

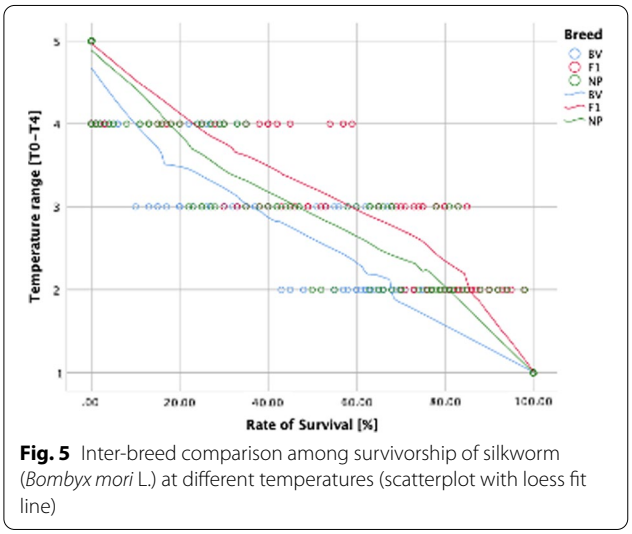

Fig. 5 Inter-breed comparison among survivorship of silkworm (*Bombyx mori* L.) at different temperatures (scatterplot with loess fit line)

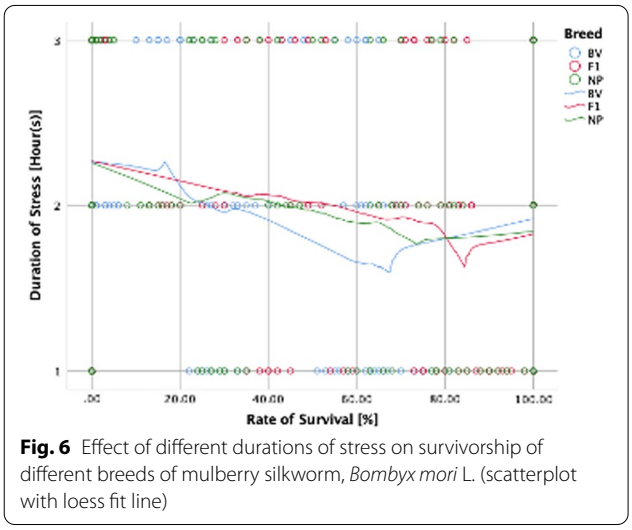

Fig. 6 Effect of different durations of stress on survivorship of different breeds of mulberry silkworm, *Bombyx mori* L. (scatterplot with loess fit line)

overall survivorship of three different breeds of mulberry silkworm (*Bombyx mori* L.) as portrayed in Fig. 6 seemed not to be as straightforward as the effect of temperature, as is evident from the presence of several crests and troughs which resulted in inter-breed variation in the survivorship.

Plasticity of upper lethal limits

The shift in upper lethal limits in mulberry silkworm for different treatment groups exhibited a heterogeneous variation across different breeds (Table 6, Fig. 4). The ULT_{50} in F1 hybrid silkworms varied within the range of 37 °C to 44 °C (Fig. 4). D3 larvae treated for 3 h duration following the plunging assay method accounted for the least ULT_{50} value, whereas the two-way ramping method yielded the maximum ULT_{50} value when F1 silkworms were treated for 1 h duration on the fourth day of the fifth instar stage (Table 3). The range of ULT_{25} in the F1 hybrid was computed to 40–47 °C (Fig. 4). The least ULT_{25} value was computed during the plunging assay when day 2 larvae of the fifth instar stage were treated for 3 h duration. However, unlike the ULT_{50} estimate, the ULT_{25} in F1 reached its maximum when day four larvae were treated for 1 h duration following the one-way ramping assay, although showing non-significant variation from that during the two-way ramping assay protocol (Table 3). The bivoltine breeds differed from the F1 hybrids in ranges of both the upper lethal temperature estimates. The ULT_{50} in bivoltine varied within the lower limit of ~ 35.2 °C which was computed in plunging lethal assay method conducted with the day 6 bivoltine larvae of fifth instar stage treated for 3 h duration and the upper limit of ~ 41.2 °C computed during the two-way ramping experiments with the day 4 larvae and 1 h treatment duration. The ULT_{25} values in the bivoltine breed were computed to be within the range of 37 °C and 45 °C. Day 6 bivoltine larvae during plunging assay method for 3 h duration accounted for the least ULT_{25} value, whereas day 4 larvae when treated for a 1-h duration in two-way ramping method yielded the maximum ULT_{25} in the bivoltine breed (Table 3, Fig. 4). In the multivoltine breed Nistari plain, the upper lethal temperatures

Table 6 The pairwise comparison result of the upper lethal temperature data in Table 3 by two-way ANOVA and the Tukey test, showing intra-breed variation in the thermal tolerance plasticity of mulberry silkworm (*Bombyx mori* L.)

Breeds	Variable	D1			D2			D3			*p*-value		
		S1	S2	S3	S1	S2	S3	S1	S2	S3	Day	Stress	D◊S[1]
F1	ULT50	42.8 ± 0.227^{b}	40.5 ± 0.217^{d}	38 ± 0.213^{fg}	43.8 ± 0.244^{a}	41.3 ± 0.186^{cd}	38.9 ± 0.214^{ef}	42.2 ± 0.189^{bc}	39.4 ± 0.237^{e}	37.6 ± 0.152^{g}	< 0.001	< 0.001	0.37
	ULT25	46 ± 0.113^{b}	43.7 ± 0.199^{d}	40.6 ± 0.242^{fg}	47 ± 0.0549^{a}	44.7 ± 0.177^{c}	41.3 ± 0.235^{f}	45.6 ± 0.152^{bc}	42.6 ± 0.26^{e}	40 ± 0.17^{g}	< 0.001	< 0.001	0.191
BV	ULT50	39.9 ± 0.213^{ab}	37.1 ± 0.205^{cd}	36.3 ± 0.207^{de}	40.8 ± 0.232^{a}	37.8 ± 0.178^{c}	36.7 ± 0.204^{d}	39.2 ± 0.18^{b}	36.9 ± 0.229^{cd}	35.4 ± 0.146^{e}	< 0.001	< 0.001	0.313
	ULT25	43.8 ± 0.221^{ab}	39.9 ± 0.311^{cd}	38.6 ± 0.315^{de}	44.8 ± 0.202^{a}	40.6 ± 0.273^{c}	39.2 ± 0.31^{cd}	43.3 ± 0.224^{b}	39.7 ± 0.362^{cd}	37.6 ± 0.238^{e}	< 0.001	< 0.001	0.56
NP	ULT50	41.2 ± 0.22^{b}	38.9 ± 0.209^{c}	37.1 ± 0.209^{d}	42.6 ± 0.262^{a}	40.4 ± 0.184^{b}	38.4 ± 0.21^{c}	40.4 ± 0.183^{b}	38.3 ± 0.231^{c}	36.3 ± 0.145^{d}	< 0.001	< 0.001	0.988
	ULT25	44.5 ± 0.178^{ab}	42.1 ± 0.241^{c}	39.6 ± 0.274^{e}	45.3 ± 0.108^{a}	43.6 ± 0.196^{b}	40.9 ± 0.248^{d}	44.1 ± 0.199^{b}	41.6 ± 0.291^{cd}	38.8 ± 0.198^{e}	< 0.001	< 0.001	0.317

Analysis done on upper lethal temperature data obtained from two-way ramping assay method. Values are means ± SEM, $n = 3$ per treatment group. $^{a-e}$Means in a row without a common superscript letter differ ($p < 0.05$) as analysed by two-way ANOVA and the Tukey test. 1D ◊ S = Day ◊ Stress interaction effect

were computed to be within the range of 36–43 °C for ULT_{50} and between 38 and 45 °C for ULT_{25} (Fig. 4). Fifth instar day 6 Nistari plain larvae treated for 3 h duration in plunging assay method exhibited the least ULT_{50}, and day 4 larvae during 1-h treatment following the two-way ramping assay accounted for the maximum ULT_{50} value. The least ULT_{25} value in the Nistari plain was computed when day 6 larvae were exposed to stress for 3 h following the plunging assay, whereas the maximum ULT_{25} value was reached during the two-way ramping assay when the day 4 larvae were treated for the 1-h duration (Table 3).

The pairwise comparison of the upper lethal temperatures across different treatment groups using the two-way ANOVA followed by Tukey–Kramer post hoc tests (HSD) revealed an intra-breed as well as an inter-breed variation (Tables 4, 6, Fig. 4). The F1 hybrid exhibited significantly higher mean survivorship than the bivoltine breed concerning both the ULT_{50} and ULT_{25} values (Table 4), whereas no significant variation was observed in the mean ULT_{50} values between the F1 hybrid and the Nistari plain breed, which, however, differed significantly from the bivoltine breed. When comparing the mean ULT_{25} values, all three breeds differed significantly from each other, with the F1 hybrid exhibiting the highest mean ULT_{25} value, followed by the Nistari plain breed and then by the bivoltine breed, as was evident from the one-way ANOVA results (Table 4). The mean upper lethal temperatures in F1 hybrids differed significantly across all the treatment groups. This observation stands right in the case of both the ULT_{50} and ULT_{25} values (Table 6). The ULT_{50} values in the bivoltine breed deviated significantly across most of the treatment groups, except the D1S2 and D3S2 treatment sets. When it comes to ULT_{25} values in the bivoltine, except for D1S2, D2S3, and the D3S2 treatment sets, the rest of the values deviated significantly across the different groups, as was revealed by the two-way ANOVA results (Table 6). The trend in the multivoltine breed Nistari plain, as analysed by the two-way ANOVA test, differed from the rest of the breeds. No significant variation in the ULT_{50} values was observed between D1S1, D2S2, and D3S1 sets; similarly, ULT_{50} values for D1S2, D2S3, and D3S2 sets did not differ significantly, and further, no significant variation was observed between D1S3 and D3S3 in terms of ULT_{50} values. When it comes to ULT_{25} in Nistari plain breed, the two-way ANOVA test result revealed that D1S3 and D3S3 did not differ significantly from each other; similarly, the deviation between D2S2 and D3S1 was not significant, and the rest of the ULT_{25} values in Nistari plain breed differed significantly from each other (Table 6).

Discussion

Among the three different assay protocols followed in the present study (Fig. 1), the direct plunging method seemed to be most adverse for the survival of all three breeds of mulberry silkworm, whereas survivorship was recorded to be maximum in the two-way ramping method across all three breeds. The effect of one-way ramping on the proportion of surviving individuals across all the experimental groups was found to be more favourable than the direct plunging assay and less beneficial compared to the two-way ramping (Table 3). These findings are in evident conformity with previous experiments on insect survival (Chidawanyika & Terblanche, 2011; Chown et al., 2009; Mitchell & Hoffmann, 2010; Nguyen et al., 2014; Powell & Bale, 2004; Terblanche et al., 2007), but at the same time contradicted the findings of Terblanche et al. (2008), in which the researchers reported a decline in the Tsetse fly survival during the ramping method. Sudden change in ambient temperature during the plunging protocol might be the reason behind the decrease in survivorship than that compared to the other two methods. Between the two ramping methods, the one-way ramping might pose thermal stress when silkworm larvae were directly returned to the basal temperature after the treatment period was over; on the other hand, the two-way ramping protocol could also have been stressful for the silkworm larvae as the extra ramping step during the gradual decrease of assay temperature might impart compound stress on the test organisms (Nguyen et al., 2014). But in reality the survivorship during the two-way ramping assay method was maximum across almost all experimental groups, and might be considered sufficient to establish the closeness of this method to natural climatic change. However, the difference in silkworm survivorship among the three assay protocols did not seem to be statistically significant, as was evident from the GLM result (Table 1). A similar finding was reported by other researchers, in which no significant variation in survivorship of test organisms was observed across different assay protocols during high-temperature tolerance experiments in DBM (Nguyen et al., 2014).

The effect of different lethal assay techniques on the upper lethal limit of mulberry silkworm seemed to exhibit a similar trend as the survivorship. A higher range of lethal temperatures (ULT_{50} and ULT_{25}) was computed across almost all experimental groups during the two-way ramping assay method, followed by the one-way ramping method and then by the direct plunging method. The difference in thermal limit depending on the assay method was also reported by various other researchers (Chown et al., 2009; Terblanche et al., 2007). The shift in upper lethal limits across different assay protocols can be attributed to the inherent genetic and physiological

characteristics of the test organism, fatigue during longer assay duration, and various other uncontrollable factors (Rezende et al., 2011). However, the impact of different assay methods on the upper lethal limits of silkworms did not seem to be statistically significant (Table 5). Therefore, cautious decisions should be made during selecting the assay method of choice for any experimental setup involving insect thermal tolerance, keeping in mind the desired implication of the sub-lethal dose to elicit a specific physiological response (Santos et al., 2011).

The thermal tolerance plasticity in mulberry silkworms demonstrated a significant inter-breed variation (Table 3, Figs. 2, 3, and 4). However, the F1 hybrid was reported to have significantly high upper lethal temperature values (ULT_{50} and ULT_{25}) compared to the other two breeds (Table 4, Fig. 4). The selected bivoltine breed (SK6 X SK7 hybrid) should usually perform well in temperate conditions (Buhroo et al., 2017) and multivoltine silkworm breeds reared in tropical conditions were shown to be slightly more thermotolerant and adjusted well with the tropical climate (Chatterjee & Ray, 2020; Hsieh et al., 1995; Rahmathulla, 2012). The F1 hybrids were reported to perform better than both the parents (Gamo & Hirabayashi, 1983; Kumar et al., 2001). These studies further stressed the findings of the present study.

Thermal tolerance, as a trait, is meticulously orchestrated by the inherent genetic constitution of an organism. Thermal preference is hypothesized to affect the evolution of an organism's thermal tolerance and thermal optimum (Calabria et al., 2012). Moreover, thermal acclimation of an organism depends on its thermal plasticity, acquired throughout the lifetime of an individual through a vast array of physiological adjustments, ultimately resulting in the alteration of gene expression (Hoffmann et al., 2003). Developmental acclimation and hardening treatments were found to influence thermal plasticity in *Drosophila* (Heerwaarden et al., 2016). The mulberry silkworms reared for the present experiments at the Sericulture Research Laboratory, Post Graduate Department of Zoology, Hooghly Mohsin College, during the extended Indian summer months (April–August), can be considered as summer acclimated. In the absence of any thermal pre-treatment in the experimental protocol, any inter-breed variation in survivorship and thermal tolerance can be attributed to the inherent thermal plasticity and developmental acclimation acquired during the rearing period. Researchers reported significant associations between the developmental temperature and both critical thermal limit and hardening capacity, which is considered an estimate of the extent of plasticity. An increase in developmental temperature was shown to increase the critical thermal limit at the cost of reduced hardening capacity (Heerwaarden et al., 2016). Moreover, slower heating methods adopted during the ramping assay techniques might induce a hardening response in certain organisms because of the pre-exposure at a lower temperature for a longer duration (Hoffmann et al., 2003). Therefore, the combined effects of inherent thermal plasticity kindled by the developmental acclimation during summer rearing and ultimately varied level of hardening (if any) elicited during the different fast and slow heating assay protocols brought about the reported variations in the survivorship and ultimately in the upper lethal spectrum of the three different breeds of mulberry silkworm (*Bombyx mori* L.).

Conclusions

Based on the findings of the present study regarding the survivorship and upper lethal temperatures, it seemed that day 4 mulberry silkworm larvae of the fifth instar stage are most suitable for thermal tolerance experiments. A similar finding was also reported by other researchers working on silkworm thermotolerance (Joy & Gopinathan, 1995). Among the durations of stress, 1–2 h duration was found to be ideal. The mean upper lethal temperature (ULT_{25}) for these three locally available breeds of mulberry silkworm (*Bombyx mori* L.) was computed to be around 47 °C, based on which the sub-lethal thermal stress must be drafted, whereas the mean upper lethal limit (ULT_0) for all three breeds was computed to be ~ 49 °C. A large number of insects are known to exhibit a ULT_{25} range between 40 and 50 °C, which, however, can vary depending on different species and different habitats (Chapman, 1998). Although the different assay methods were found to exert a non-significant impact on silkworm survivorship, still, owing to the maximum survivorship across almost all the experimental groups, the two-way ramping lethal assay technique involving gradual increase and decrease in temperatures must be considered to be ideal for silkworm thermal tolerance and heat-shock experiments. Moreover, the thermal tolerance plasticity in mulberry silkworm (*B. mori*) was found to exhibit a significant breed-specific variation. The result of the study must strictly be considered viable only when the aforementioned experimental criteria are met. Therefore, similar experiments conducted at a different clime may exhibit a change in the recorded upper lethal temperature values for each breed. Thermal lethality in insects across different assay methods was pointed out to be unpredictable by Rezende et al. (2011) and Santos et al. (2011). Therefore, the choice of specific assay protocols should be a precisely calculated decision. In conclusion, many more endeavours similar to the present one during different seasons and involving different test organisms must be undertaken to reinforce our archaic understanding of thermal plasticity.

Abbreviations
ANOVA: Analysis of variance; BV: Bivoltine breed; DBM: Diamondback moth; F1: F1 hybrid; GLM: Generalized linear model; HSD: Honestly significant difference; NP: Nistari plain; SEM: Standard error of mean; UGC: University Grants Commission; ULT: Upper lethal temperature; ULT_0: Upper lethal temperature 0; ULT_{25}: Upper lethal temperature 25; ULT_{50}: Upper lethal temperature 50.

Supplementary Information

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Additional file 1. Variation in daily average temperature [ºC] during the extended summer months (April-October)-2016-2019.

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Author contributions
MC was corresponding author, contributed to conceptualization, methodology, writing—original draft preparation, data curation, and visualization, and provided software. NR was involved in conceptualization, methodology, investigation, supervision, and writing—reviewing and editing. All authors have read and approved the manuscript.

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Availability of data and materials
The authors declare that [the/all other] data supporting the findings of this study are available within the article [and its Additional file 1].

Declarations

Ethics approval and consent to participate
Not applicable; as mulberry silkworm is a cultured insect, it does not come under the purview of the animal ethical committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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