COMPARATIVE SUSCEPTIBILITY OF *CHIRONOMUS* AND *DROSOPHILA* TO EXPOSURE TO EACH AND COMBINATIONS OF THE FOLLOWING STRESSORS: DESICCATION, HEAT STRESS AND STARVATION

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ABSTRACT

In natural ecosystems, organisms are usually subject to environmental stress. In order to understand the response to a combination of three stressors (desiccation, heat stress and starvation), two dipteran insects, *Chironomus ramosus* (aquatic) and *Drosophila melanogaster* (terrestrial) were chosen, the former being more primitive than the latter. The mortality level as a function of the duration of the exposure to stress revealed that these two evolutionarily distinct and ecologically diverse insects differ in their response. Interestingly, when the tolerance thresholds of *C. ramosus* and *D. melanogaster* to single and multiple stressors was compared, a synergistic effect was recorded with much higher levels mortality occurring when subjected simultaneously to several stressors. *Chironomus* larvae were more vulnerable than *Drosophila* larvae when subjected to all three stressors simultaneously. The findings of this pilot study indicate the ecological risk for macro-invertebrate biota posed by adverse environmental conditions.

Keywords: *Chironomus*; desiccation stress; *Drosophila*; heat stress; multiple stress; starvation stress

Introduction

In nature, organisms experience changes in environmental conditions, such as heat stress, starvation, desiccation, osmotic imbalance, hypoxia, radiation, etc. Often, organisms exhibit specific patterns of adaptations in response to these environmental stresses (Bijlsma and Loeschcke 2005; Davies et al. 2014). In this context, insects are suitable model systems for determining the responses to environmental stress (Datkhile et al. 2015; Thorat et al. 2016, 2017). This is because in spite of being small and having a high metabolic rate, insects are among the most widely distributed animals and occur in almost all of the habitats in the biosphere and as a consequence are adapted to cope with a wide range of environmental stressors.

Effect of a combination of different stressors on organisms inhabiting diverse environmental conditions is of prime concern in the context of climate change. There is currently no in-depth study that address the adaptive response of insects to stressors. According to the 'Stress-Exposure-Response' (SER) model, abiotic stressors affect different species in variety of ways (Freedman 2015). Organisms have adapted to thrive and cope with multiple stressors acting simultaneously. The strategies used by insects to survive multiple stressors warrant more fundamental level studies, which is the rationale behind the present study. For this work, two phylogenetically distant and ecologically diverse insects, namely *Chironomus ramosus* and *Drosophila melanogaster,* were chosen. The former is an aquatic and the latter a terrestrial insect. We investigated the responses of these two insects to single and multiple stressors. Since survival depends on the length of exposure to stressors (Schulte 2014), we also looked into the temporal aspect of the response to stress. Our study revealed a striking difference in the responses to the exposure to specific stressors and several stressors simultaneously.

Methods

Rearing and maintenance of cultures

C. ramosus was mass reared in specially designed netted cages at a temperature of 24 ± 2 °C as described earlier (Nath and Godbole 1998). Early fourth instar larvae were used in all the experiments. An inbreed population of *D. melanogaster* (ORK strain) was maintained as described earlier by Thorat et al. (2016) in a BOD incubator set at 24 ± 2 °C.

Experimental Design

Ten larvae of either *D. melanogaster* or *C. ramosus* were used in each experiment, in which the larvae were exposed to either desiccation, heat stress or starvation, or combinations of these stressors for different durations or until 100% mortality was recorded. Each experiment was replicated ten times.

Desiccation

Five hundred grams of silica gel was added to the desiccating chamber 12 hrs prior to its use in order to obtain a value of <5% relative humidity (RH), which was monitored using a hygrometer. Larvae of *C. ramosus* and *D. melanogaster* were desiccated in this chamber on dry tissue paper placed in a glass Petri dish. The time to when

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100% had died was recorded. Larval survival was judged on the basis of either abdominal contractions (*Drosophila*) or undulatory movements (*Chironomus*) when gently poked with a blunt needle. Untreated larvae were used as a control. Each experiment was replicated ten times.

Heat stress

Heat stress was administered by transferring larvae of both *D. melanogaster* and *C. ramosus* to incubators at 37 °C and 40 °C, respectively, in order to subject them to heat stress. Induction of HSP70 as well as responses of *Chironomus* (Nath and Lakhotia 1989; Nath and Gharpure 2015) and *Drosophila* (Lindquist 1980) were the criteria used in the present study for indicating heat stress, which indicates it occurs at 40 °C in *C. ramosus* and 37 °C in *D. melanogaster*.

Starvation

Larvae of both *D. melanogaster* and *C. ramosus* were removed from the rearing media, carefully cleaned and then starved to death. Since Chironomid larvae need to be able to burrow into a substrate they were provided with a substrate consisting of inert sand particles as described by Naik et al. (2006).

Multiple stressors

Both larvae of *D. melanogaster* and *C. ramosus* were exposed to either desiccation (D), heat stress (H) or starvation (S) either in a combination of two (D + S; D + H; H + S) or three stressors (D + H + S) simultaneously. The time to when all the larvae had died was monitored for each combination.

Data analysis

Probit analysis of the percentage mortality data was carried out. The mean mortality ± SE values were analysed statistically followed by ANOVA. Percentage mortality of treated samples were normalized relative to that of the control (without stress) using Abbott's formula (Abbott 1925). The slope of the regression line resulting from the probit analysis was determined for each experiment and goodness of fit was assessed using Chi-square tests.

Results and Discussion

We determined the differences in the responses of larvae of both *D. melanogaster* and *C. ramosus* in terms of

Table 1 The LT_{20}, LT_{50} and LT_{90} values predicted by the Probit analysis of the results of different stress treatments: A) *Drosophila melanogaster*, B) *Chironomus ramosus*.

Stress	LT_{20} (Hrs)	Fiducial limits of LT_{20} value	LT_{50} (Hrs)	Fiducial limits of LT_{50} value	LT_{90} (Hrs)	Fiducial limits of LT_{90} value	Slope ± SE[b]	χ^2	d.f.[a]
A. *Drosophila melanogaster*									
Desiccation	8.092	7.520–8.707	9.494	8.823–10.016	11.391	10.889–11.767	12.131 ± 0.016	0.904	2
Heat stress	10.483	9.819–11.193	12.927	12.107–13.801	17.784	16.657–18.987	9.334 ± 0.015	0.994	9
Starvation	25.520	24.561–26.517	30.263	29.098–31.416	39.139	37.668–40.668	11.505 ± 0.008	1.000	20
Desiccation + Starvation	8.092	7.520–8.707	9.494	8.823–10.016	11.391	10.889–11.767	12.131 ± 0.016	0.904	2
Desiccation + Heat stress	3.428	3.045–3.860	4.387	3.897–4.940	6.388	5.673–7.193	7.931 ± 0.026	0.775	3
Heat stress + Starvation	8.502	8.064–9.385	11.004	10.198–11.873	15.733	14.580–16.977	8.304 ± 0.017	1.000	8
Multiple Stressors (D + H + S)	3.428	3.045–3.860	4.387	3.897–4.940	6.388	5.673–7.193	7.931 ± 0.026	0.775	3
B. *Chironomus ramosus*									
Desiccation	0.837	0.794–8.707	0.937	0.888–10.016	1.049	1.008–1.767	17.534 ± 0.016	0.884	3
Heat stress	14.691	13.735–15.712	22.319	20.868–23.871	42.193	39.450–45.127	4.679 ± 0.015	1.000	39
Starvation	49.778	48.229–51.378	62.899	60.940–64.922	89.820	87.022–92.708	8.323 ± 0.007	1.000	56
Desiccation + Starvation	0.837	0.794–8.707	0.937	0.888–10.016	1.049	1.008–1.767	17.534 ± 0.016	0.884	3
Desiccation + Heat stress	0.601	0.592–0.669	0.705	0.663–0.749	0.820	0.788–0.890	17.099 ± 0.014	0.804	2
Heat stress + Starvation	8.332	7.614–9.117	12.349	11.286–13.513	22.484	20.577–24.602	4.966 ± 0.020	1.000	19
Multiple Stressors (D + H + S)	0.601	0.592–0.669	0.705	0.663–0.749	0.820	0.788–0.890	17.099 ± 0.014	0.804	2

[a] Degrees of freedom; [b] Standard error; χ^2 = Chi-square value; LT_{20} = Length time required to kill 20% of the larvae; LT_{50} = Length of time required to kill 50% of the larvae; LT_{90} = Length of time required to kill 90% of the larvae.

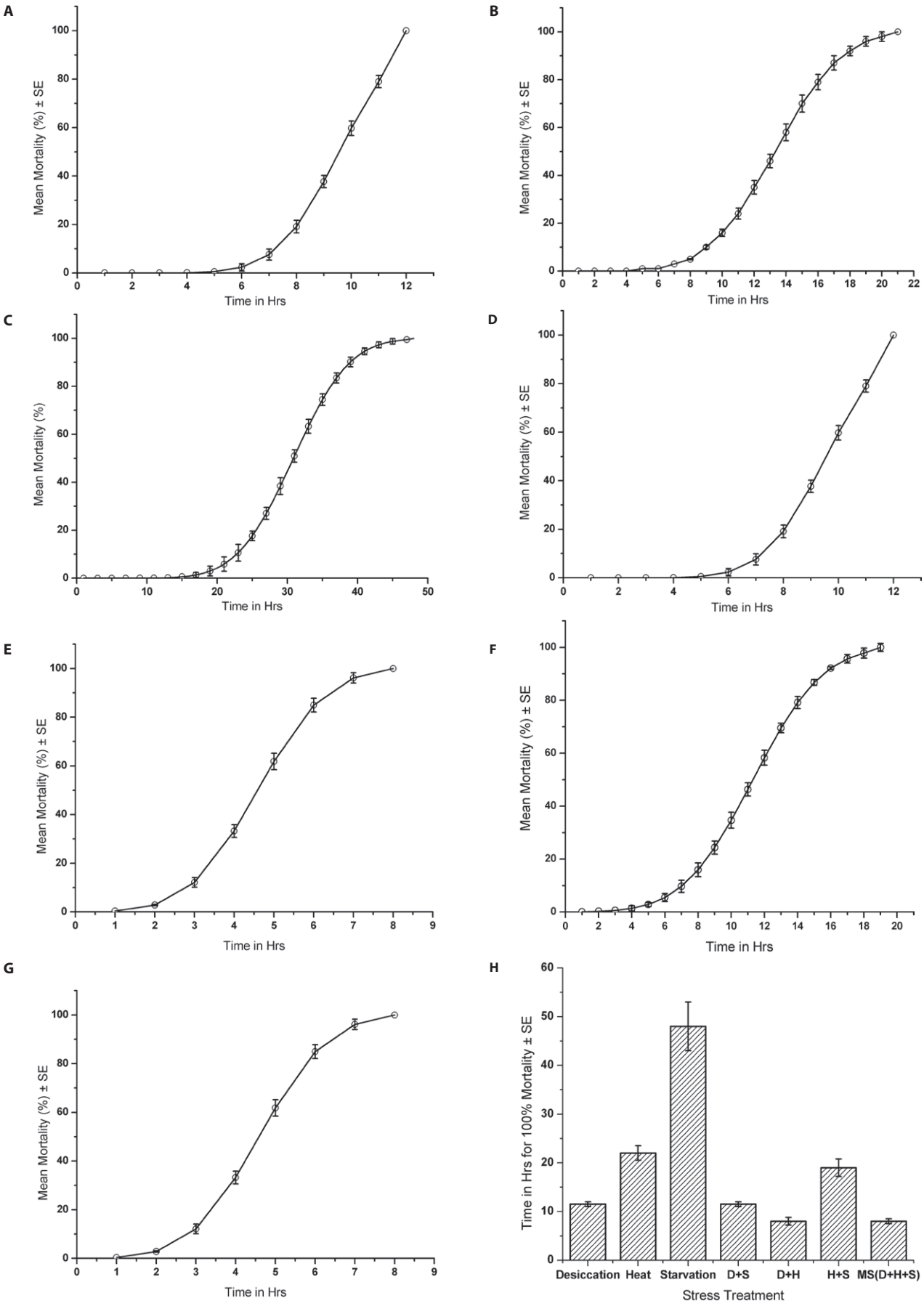

Fig. 1 Curve of the mean mortality recorded over time (hours) of *D. melanogaster* larvae exposed to a) desiccation, b) heat stress, c) starvation, d) desiccation + starvation, e) desiccation + heat stress, f) heat stress + starvation, g) multiple stressors: desiccation + heat stress + starvation, h) single and multiple exposure to stressors. F (6, 28) = 355.87, $P < 0.001$; ANOVA was used to compare the results for the different stress treatments.

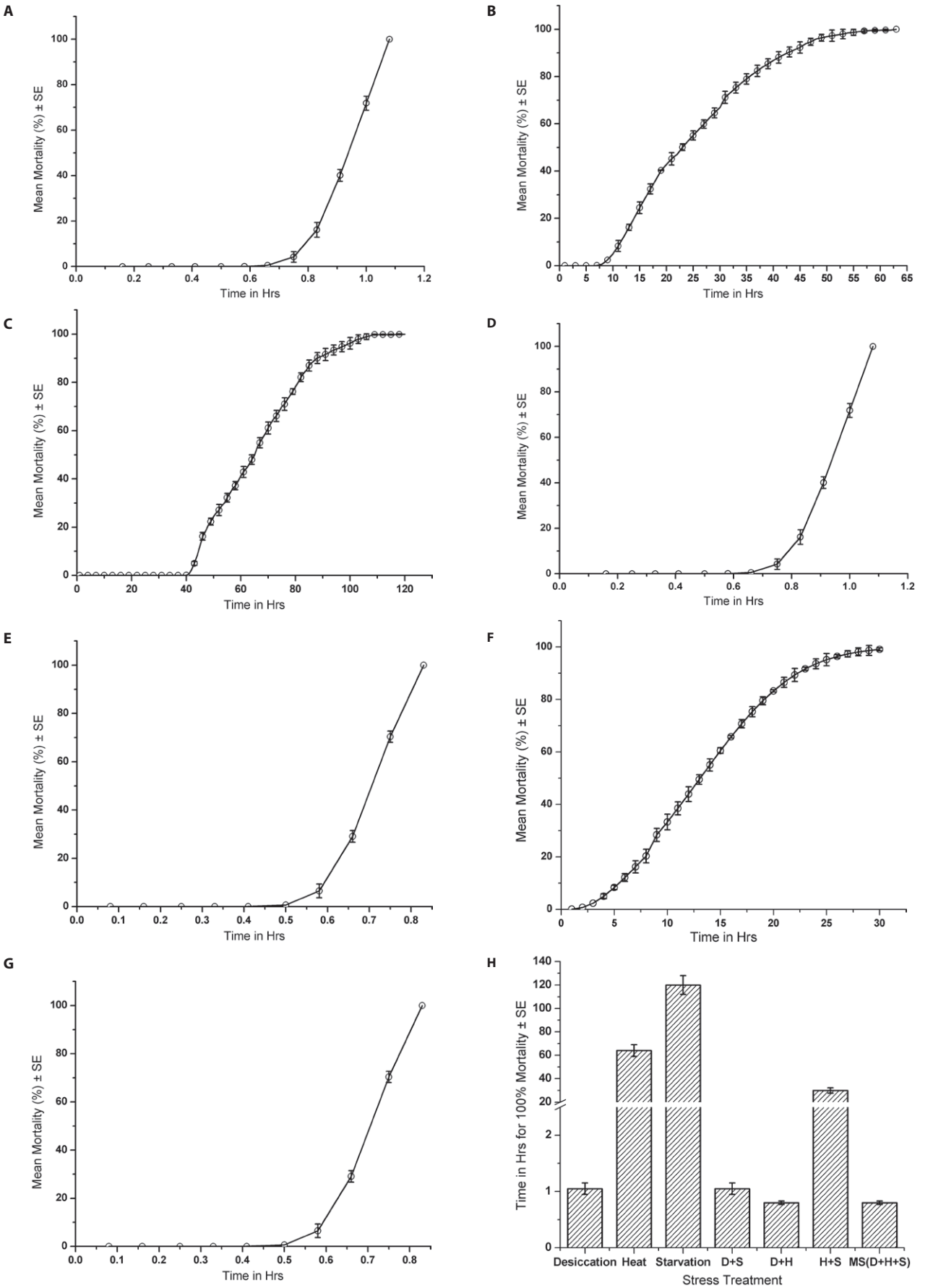

Fig. 2 Curves of the mean mortality recorded over time (hours) of *C. ramosus* larvae exposed to a) desiccation, b) heat stress, c) starvation, d) desiccation + starvation, e) desiccation + heat stress, f) heat stress + starvation g) multiple stressors: desiccation + heat stress + starvation, h) single and multiple exposure to stressors. F (6, 28) = 2388.7, $P < 0.001$; ANOVA was used to compare the results of the different stress treatments.

mortality due to desiccation, heat stress and starvation when subjected to these stressors separately or in combination. *D. melanogaster* showed a steady increase in morality with increase in the time the larvae were exposed to stress, irrespective of the type of stress. Its larvae survived an exposure to desiccation of 11.5 ± 0.5 hrs and 22 ± 1.5 hrs to heat stress and were even more tolerant of starvation as they survived for 48 ± 5 hrs. In the multiple stress treatments, they survived for11.5 ± 0.5 hrs in the D + S treatment, 8 ± 0.8 hrs in the D + H and 19 ± 1.8 hrs in the H + S treatment. The larvae of *D. melanogaster* were significantly less tolerant (8 ± 0.5hrs) of simultaneous exposure to several stressors, i.e. D + H + S, than a single stressor (Fig. 1). The time required to kill 20% (LT_{20}), 50% (LT_{50}) and 90% (LT_{90}) of the larvae was determined using Probit analysis (Table 1A).

The mortality of the larvae of *C. ramosus* also increased with the length of time they were exposed to the stressors. The larvae survived 1.05 ± 0.1 hrs of desiccation, 64 ± 5 hrs heat stress and 120 ± 8 hrs of starvation. In the multiple stress treatments, larvae survived 1.05 ± 0.1 hrs exposure to D + S, 0.8 ± 0.03 hrs to D + H and 30 ± 2.3 hrs to H + S. The larvae were significantly less tolerant of exposure to D + H + S, as they only survived for 0.8 ± 0.03 hrs (Fig. 2). Comparison of the results of single and multiple stress treatments revealed that the larvae of *C. ramosus* were significantly less tolerant of simultaneous exposure to several stressors than a single stressor. The time required to kill 20% (LT_{20}), 50% (LT_{50}) and 90% (LT_{90}) of the larvae was determined using Probit analysis (Table 1B).

Of the three stressors used, both *C. ramosus* and *D. melanogaster* were most vulnerable to desiccation followed by heat stress. This corroborates an earlier hypothesis that tolerance of desiccation determines the distribution of insects (Kellermann et al. 2009) along with the way insects cope with heat stress combined with a shortage of water (Chown et al. 2011). Nevertheless, starvation affected both the insects tested the least. Therefore, despite the differences in their ecology and evolution these two insects responded similarly to the stressors.

Interestingly, *D. melanogaster* was relatively more tolerant than *C. ramosus* when subjected to all three stressors simultaneously. This difference in the tolerance of terrestrial and aquatic insect of abiotic factors was previously reported by Chown et al. (2015). Nevertheless, aquatic insects experience less variation in their thermal environment than terrestrial insects, therefore, it is not surprising that aquatic insects are more sensitive to heat stress. Furthermore, desiccation affects aquatic Chironomid midge larvae more than the terrestrial Drosophilid larvae as is reported earlier by Thorat et al. (2017).

In the past, although concerns were raised about two-species comparisons (Garland and Adolph 1994), they were addressed by choosing model organisms with markedly different ecologies and using a more rigorous experimental protocol. Interestingly, although the organisms studied belong to same order they differ markedly in their ecology and evolutionary history.

According to Jorgensen (2010), organisms exposed simultaneously to multiple stressors may respond synergistically, additively or antagonistically. The findings of present study clearly indicate that the larvae of both *C. ramosus* and *D. melanogaster* show a synergistic response to a simultaneous exposure to several stressors, as such an exposure has a much greater effect than exposure to a single stressor.

A recently published meta-analysis of the status of multi-stress research on aquatic organisms indicates a lack of general consensus on their value in risk assessment and an appropriate scientific framework (Noges et al. 2016). In this context, our study is the first attempt, although on a small scale, to determine the effect on two species of insects of exposure to either a single stressor or a simultaneous exposure to several stressors.

Conclusions

The present study clearly indicate that the thresholds of tolerance of *C. ramosus* and *D. melanogaster* of desiccation, heat stress or starvation either on their own or combined differed. The effects of starvation, desiccation and heat stress are similar and future studies may throw light on the cellular and molecular basis of this commonality as well as the uniqueness of stress-signalling pathways when organisms are exposed simultaneously to many stressors. The results of this study provide a valuable insight into how to carry out ecological risk assessment programmes.

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