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RESEARCH ARTICLE

Stress-induced plastic responses in *Drosophila simulans* following exposure to combinations of temperature and humidity levels

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SUMMARY

Plastic responses to heat and desiccation stress in insects have been studied in many laboratory experiments on *Drosophila*. However, in these studies the possible interaction between the corresponding stress factors in natural environments has not been taken into consideration. We investigated changes in heat and desiccation resistance of adult *Drosophila simulans* after short-term exposures to different temperatures (35, 31 and 18°C) in combination with high and low relative humidity (*ca.* 90 and 20%, respectively). Hardening under extreme conditions (35 or 31°C and low relative humidity) commonly resulted in higher resistance to heat and desiccation as compared with other less stressful combinations of temperature and humidity levels. The concentration of the heat-shock protein Hsp70 in the experimental flies increased following almost all applied treatments. Life span of the hardened flies under non-stressful conditions was reduced irrespective of the stress dose, indicating a fitness cost for the plastic responses. The results of the study show that hardening using combined heat and desiccation stress can be very efficient with regard to induction of plastic responses improving tolerance to both types of stress. This may favour adaptation to hot and dry climatic conditions, though the negative effects on fitness are likely to constrain evolution of such plastic responses.

Key words: insect, heat stress, desiccation stress, hardening, stress resistance, heat-shock proteins, longevity.

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INTRODUCTION

The abundance and distributional patterns of terrestrial arthropods are strongly dependent on the ability of these organisms to tolerate thermal extremes and the loss of water (Hoffmann and Blows, 1994; Addo-Bediako et al., 2000; Chown and Nicolson, 2004; Chown et al., 2011). Therefore, thermal and desiccation resistance can be considered as important physiological traits affecting the adaptation of insects to their environments. A common form of adaptation is a plastic response induced by preliminary exposures to new and/or stressful conditions that allows an organism to enhance its stress resistance. In physiology, long-term exposures (days and weeks) to new conditions that are within normal viable limits are usually termed ‘acclimation’, whereas short-term exposures (minutes and hours) to sub-lethal conditions are referred to as ‘hardening’ (Hoffmann et al., 2003; Bowler, 2005).

Plastic responses to thermal and desiccation stresses have been considered in various taxa of terrestrial arthropods such as springtails, locusts, ants, whiteflies, beetles, moths, parasitic wasps, and fruit and flesh flies (for reviews, see Hoffmann et al., 2003; Sinclair et al., 2003; Chown and Nicolson, 2004; Chown and Terblanche, 2006). To explain the observed effects, a few physiological mechanisms have been suggested, including induction of heat-shock proteins (Hsps), changes in membrane lipid composition, sugar or polyol concentration and metabolic rate. A large number of studies on thermal acclimation and hardening have been conducted within the *Drosophila* genus, particularly on the model organism *Drosophila melanogaster*. This species has been most extensively used in studies of Hsps, which are known for their role as molecular chaperones in the cellular stress response in diverse organisms from bacteria to humans (Lindquist, 1986; Feder and Hofmann, 1999). It has been well established that increased thermotolerance after heat hardening in *D. melanogaster* is associated with Hsps, specifically the major heat shock protein, Hsp70 (Solomon et al., 1991; Feder and Krebs, 1997; Feder and Hofmann, 1999).

The effects of acclimation and hardening by desiccation stress received primarily less attention in *Drosophila* studies, but the situation has changed in the last few years. Evidence on enhanced tolerance to water loss after dehydration treatments has been obtained for several species of *Drosophila*, though not all tested species showed such a plastic response (Hoffmann, 1990; Hoffmann, 1991; Bazinet et al., 2010; Bubliy et al., 2012a; Parkash et al., 2012a; Parkash et al., 2012b; Aggarwal et al., 2013). At the same time, data on possible physiological mechanisms for desiccation acclimation/hardening in these fruit flies remain scarce. It was recently reported that low humidity treatments lead to a reduction in cuticular water loss rate (Bazinet et al., 2010) and an increase in the level of carbohydrates (Parkash et al., 2012a).

Studies on *Drosophila* and other insects have commonly examined high temperature hardening under non-stressful humidity conditions, whereas dehydration treatments have been performed in the absence of heat stress. It is obvious, however, that for many widespread species the amount of water in the environment varies across climates with different temperature regime. Consequently, such species can face the combined effect of high temperature and desiccation stress, and their physiological plastic responses can be determined by the interaction of the corresponding environmental factors. Investigating these responses is of strong ecological importance as insects depend upon them to survive and reproduce.

Here we explored the effects of hardening on heat and desiccation tolerance in *Drosophila simulans* Sturtevant 1919, applying different temperature levels under low and high humidity conditions. *Drosophila simulans* is a close relative of *D. melanogaster*, with both species originating from the Afrotropical region (Lachaise and Silvain, 2004). Because of their exceptional colonizing ability, these two human commensals spread all over the world and can be found today in different regions from the Equator to temperate latitudes. Comparative studies on physiological traits have shown that *D. simulans* is generally more sensitive to environmental stresses, including high temperature and low humidity conditions, than *D. melanogaster* (for a review, see David et al., 2004).

In three independent experiments, we considered the following stressful treatments: (1) 35°C at low *versus* high relative humidity (RH), (2) 31°C at low *versus* high RH, and (3) 31°C *versus* 18°C at low RH. The low and high humidity conditions were represented by RH values close to 20 and 90%, respectively. Whereas extremely high humidity is typical for wet tropical climates, in many arid subtropical and temperate regions it often falls below 20% (climatic maps can be found at http://www.intellicast.com). The use of temperatures of 31 and 35°C allows testing for plastic responses, which may be associated with different physiological mechanisms. It has been shown that short-term exposures of adult *D. simulans* to temperatures of 33–37°C result in an increase in Hsp70 expression with a peak at 36°C (Krebs, 1999). However, 31°C is at the upper boundary of the normal temperature range for this species (Cohet et al., 1980; David et al., 2004) where one can expect a relatively weak Hsp70 synthesis because of a low stress level. Comparing the 31 and 18°C low humidity treatments can provide information on the efficiency of desiccation hardening at mildly stressful *versus* non-stressful temperatures.

To test for association between hardening and induction of Hsps, we assayed the level of Hsp70 expression after all treatments. Although the heat-induced Hsp70 expression in *Drosophila* has been reported in a number of studies, there is no evidence on its upregulation by desiccation stress. However, it might be expected based on the results of other insect studies (Tammariello et al., 1999; Hayward et al., 2004; Benoit et al., 2009; Lopez-Martinez et al., 2009; Benoit et al., 2010). Finally, in the first and third experiments we estimated longevity of flies from all treatments under non-stressful conditions. It has been recently shown that both heat and desiccation hardening negatively affect this life-history parameter in *D. melanogaster* (Bubliy et al., 2012a). Costs of hardening to heat stress have also been revealed in *Drosophila* for life-history traits related to reproductive functions (Krebs and Loeschcke, 1994; Tatar, 1999; Hercus et al., 2003). In contrast, some authors found hormetic effects of heat treatments on longevity (Khazaeli et al., 1997; Le Bourg et al., 2001; Hercus et al., 2003; Scannapieco et al., 2007). However, none of the above-mentioned studies considered the combined effect of temperature and desiccation stress.

MATERIALS AND METHODS

Experimental flies

Drosophila simulans were obtained from eggs collected in August 2012 near Vergato (Bologna region, Italy) using traps baited with banana and peaches. After eclosion of all flies from the eggs in the laboratory, 450 non-virgin females were placed in individual vials to start the first laboratory generation. For the second generation, 2250 pairs of their progenies (five pairs from a vial) were mixed and transferred to culture bottles. The flies were reared at 23°C under 16 h:8 h light:dark cycles on a standard oatmeal–sugar–yeast–agar *Drosophila* medium. In the third generation, *ca.* 500 pairs of young flies were sampled to establish a new mass-bred population, which was subsequently used in the present experiments. This population was maintained at 25°C under 12 h:12 h light:dark cycles as discrete generations with mixing of adults among bottles in each generation. We used 600 pairs of flies as parents for every new generation. They were reared on a standard oatmeal–sugar–yeast–agar *Drosophila* medium in plastic round-bottom bottles (60×130 mm) under uncontrolled but low larval density conditions.

All experiments were performed with virgin females from the third generation of the mass-bred population. These females were collected from the culture bottles under CO_2 anaesthesia, whereas subsequent transfers between vials were performed using aspiration without anaesthesia. The flies were kept in a constant-temperature room at 23°C and 50% RH. They were placed in plastic vials containing the standard *Drosophila* medium without live yeast. The dimension of the vials was 25×95 mm and the number of flies per vial was 20. The same type of vials was used in all treatments as well as for recovery of stressed flies and in the longevity assays. The number of individuals in the vials was always equal to 20 (but it decreased over time in the longevity assays as flies started to die). As heat and desiccation tolerance in *Drosophila* is known to decline with age (Gibbs and Markow, 2001; Sørensen and Loeschcke, 2002; Bowler and Terblanche, 2008), this parameter was strongly controlled in all our experiments.

Treatments

We conducted three independent experiments, in which different combinations of temperature and humidity levels were applied (Table 1). In the first and second experiments, the flies were exposed to temperature regimes with maximum values of 35 and

Table 1. Stressful treatments applied to *Drosophila simulans* females in three experiments

		Temperature (°C)		RH (%)		
Experiment	Treatment (abbreviation)	Range	Mean ± s.d.	Range	Mean ± s.d.	Duration (min)
1	35°C, low RH (35LH)	23.6–35.1	33.3±3.5	14.6–33.2	18.4±5.3	60
1	35°C, high RH (35HH)	23.7–35.2	33.1±3.0	49.9–89.2	84.2±10.5	60
2	31°C, low RH (31LH)	22.6–31.6	30.7±1.5	18.3–37.4	20.6±3.3	195
2	31°C, high RH (31HH)	23.2–31.2	30.5±1.4	53.9–88.6	85.0±5.3	195
3	31°C, low RH (31LH)	23.6–31.1	30.8±1.3	18.9–33.9	20.0±2.6	195
3	18°C, low RH (18LH)	17.7–22.2	18.1±0.8	14.5–28.9	17.2±3.1	195

RH, relative humidity; LH, low humidity; HH, high humidity.

Measurements were recorded with iButton loggers inside treatment vials using resolutions of 0.5°C and 0.6% for temperature and RH, respectively. Both parameters commonly approached their ultimate values (35, 31 or 18°C for temperature and 15–19% or 89% for RH, respectively) 25–30 min after the vials had been placed into the climatic chambers and remained close to these values (with minor fluctuations) until the end of treatments. For each experiment, there were also control flies kept at non-stressful conditions (23°C, 50% RH).

31°C, respectively, using both low and high RH (35LH and 35HH, 31LH and 31HH). In the third experiment, low RH was combined with two temperatures, 31 and 18°C (31LH and 18LH). The experimental design is explained by the fact that we employed two environmental chambers (cabinets) with temperature and humidity control (model Termaks KB8000F, Termaks AS, Bergen, Norway) that allowed working with only two samples of flies at a time.

For the treatments, 5.5- to 6-day-old flies were placed in empty vials covered with gauze. Racks with the vials were quickly put into the environmental chambers with the appropriate temperature and humidity settings. The variation in temperature and RH values in the experimental vials during the treatments is shown in Table 1. The data were obtained using DS1923 iButton loggers (Maxim Integrated, San Jose, CA, USA), which have a measurement accuracy (with software correction) of ±0.5°C and ±5% for temperature and RH, respectively. The temperature inside vials was also visually monitored with alcohol thermometers through glass windows in the doors of climatic chambers (data not presented). The duration of treatments (Table 1) was chosen such that in a few hours after the most stressful hardenings (recovery period) the mortality rate did not exceed 10%. The flies that were able to walk on the side of a vial when held vertical were scored as living.

After each treatment, one part of the flies was immediately frozen using liquid nitrogen and put into a freezer at –80°C. Another part was transferred to fresh vials containing standard *Drosophila* medium without live yeast. They were allowed to recover at 23°C and 50% RH before being used in the stress resistance and longevity tests. The recovery times are given in Table 2.

Stress resistance

In each experiment, flies from the stressful treatments were tested together with control flies. The resistance assays were performed using small glass vials (45×15 mm) without *Drosophila* medium under the temperature and humidity conditions shown in Table 2). Each vial contained only one fly and was attached to a metal rack placed in a 70×35×35 cm fish tank (aquarium). For the heat resistance test, the vials were tightly closed with screw plastic caps and the corresponding tank was filled with heated water. Its temperature was maintained with two thermostats (Hetto HMT200, Holm & Halby A/S, Brøndby, Denmark). Desiccation resistance was assayed in a tank containing 3 kg of SORBSIL CHAMELEON silica gel (Oker-Chemie GmbH, Goslar, Germany) and sealed using a masking tape. The testing vials were covered with gauze. In both of the above-mentioned assays, resistance was estimated as knockdown time, the time until flies inside a tank went into a coma, i.e. were unable to move any body part. The flies were checked for knockdown every minute and every hour in the heat and desiccation resistance tests, respectively. The number of flies for which we recorded knockdown times ranged from 60 to 64 per treatment.

Hsp70

The level of Hsp70 expression was estimated with the ELISA technique following the protocol of Sørensen et al. (Sørensen et al., 1999). The flies previously stored at –80°C were homogenized on ice. For each experiment, one microwell ELISA plate with 15 samples was prepared. Each treatment and control were represented on the plate by five replicates, and each replicate consisted of 12–13 homogenized flies. The samples were adjusted to equal amounts of total protein content by BCA analysis and the Hsp70 level was determined using the monoclonal antibody 7.FB, which is specific for this protein in several *Drosophila* species, including *D. simulans* (Velazquez and Lindquist, 1984; Welte et al., 1993; Krebs, 1999). The amount of Hsp70 in each sample was quantified from the intensity of an enzymatic reaction and measured as absorbance with a spectrophotometer.

Longevity

Longevity was assayed in the first and third experiments under non-stressful conditions (Table 2). After a recovery period of 18 h, the flies from the stressful treatments (see Table 1) were placed in a constant-temperature room together with non-stressed control flies of the same age. The number of flies per vial (in the beginning of the assay) and the number of vials per treatment within experiment was equal to 20. Every third day the flies were transferred into new vials containing fresh standard *Drosophila* medium without live yeast. Mortality was recorded every third day until all flies had died. For each fly, longevity was calculated as the time interval from the day of eclosion to the day when it was found dead.

Statistics

Data analysis was based on standard statistical methods implemented in STATISTICA 7.0 (StatSoft, Tulsa, OK, USA). Comparisons between samples of flies from different treatments were carried out within each experiment using the Mann–Whitney *U*-test and the obtained probability values were corrected for the number of comparisons following the sequential Bonferroni procedure (Rice, 1989).

RESULTS

Hardening at low humidity at 35 or 31°C was more stressful than other treatments, resulting in significantly higher mortality rate (comparisons with 2×2 contingency χ^2-test, data not presented). For 35LH, the proportion of dead individuals after the recovery period was 4.4% and for 31LH it was 8.0 and 9.8% in the second and third experiments, respectively. For 35HH, 31HH and 18LH the respective values were 0.1, 0.2 and 0%.

Fig. 1 shows mean values and their 95% confidence intervals for all considered traits after stressful treatments in the three experiments. In Table 3 we present results of statistical tests for comparison of these values within experiments. In all cases, more stressful conditions (35 or 31°C in combination with low humidity)

Table 2. Recovery periods for heat- and/or desiccation-stressed flies before three tests, and testing conditions

Test	Recovery period (h)	Testing conditions	
		Temperature (°C)	RH (%)
Heat resistance	10	38.2	30–50
Desiccation resistance	9	25	Close to 0
Longevity	18	25	50

Stressed flies recovered at 23°C and 50% RH on standard culture medium and were then tested together with non-stressed control flies.
In the heat resistance test, RH inside testing vials gradually decreased.

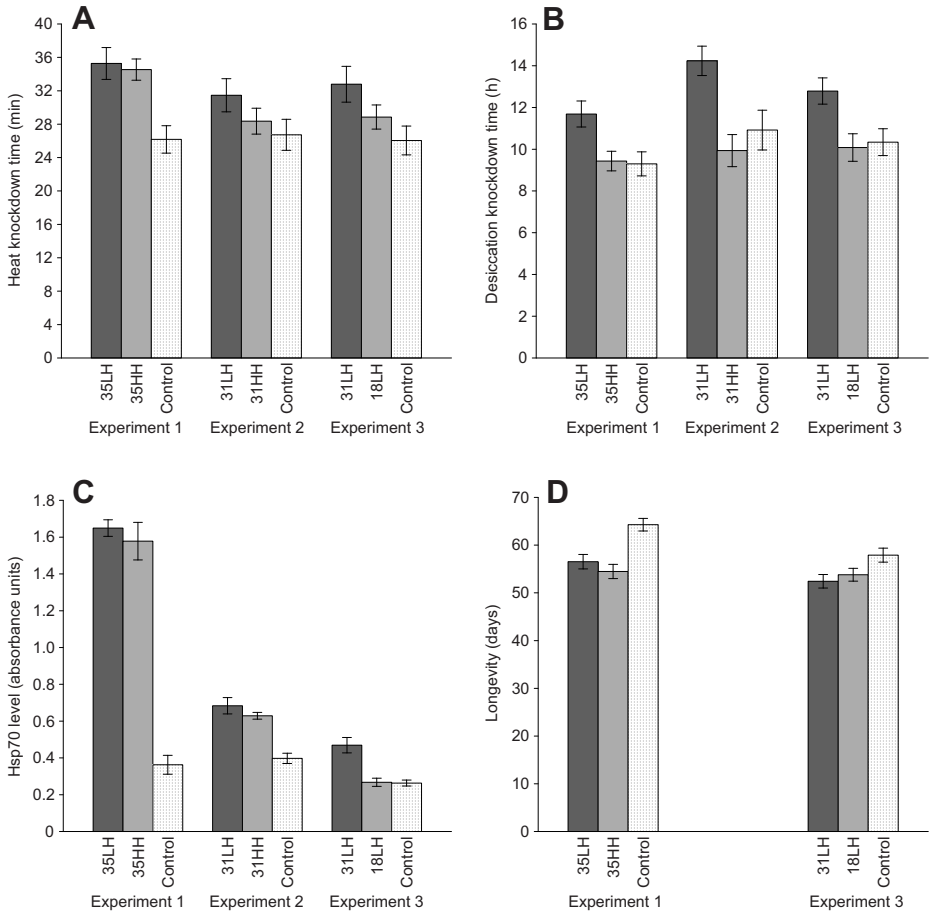

Fig. 1. Mean values and their 95% confidence intervals for heat knockdown time (A), desiccation knockdown time (B), Hsp70 level (C) and longevity (D) in *Drosophila simulans* females following treatments with different combinations of temperature and humidity levels. For treatment abbreviations, see Table 1.

resulted in increased resistance as compared with the control flies (Fig. 1A,B, Table 3). Milder stress affected only heat resistance: heat knockdown time in the 35HH and 18LH treatments was greater than in the respective controls. In all comparisons except 35LH *versus* 35HH for heat resistance, we found that flies hardened at highly stressful conditions were more resistant both to heat and desiccation. The level of Hsp70 was increased as compared with the controls by all stressful treatments except 18LH, and the increase was more pronounced in the first experiment, where the flies were exposed to 35°C (Fig. 1C; Table 3). At this temperature, there was no statistically significant difference between the low and high humidity conditions, whereas at 31°C the desiccation-stressed flies showed a higher level of Hsp70 than those exposed to high RH. Under low humidity conditions, the Hsp70 level was higher at 31°C than at 18°C. The longevity assays, performed in the first and third experiments, showed that the hardened flies had a shorter life span than the control flies and there was no difference between the hardening treatments within experiments (Fig. 1D, Table 3).

DISCUSSION

Results of the present study demonstrate that treatments using combined heat and desiccation stress can be more efficient with regard to improving stress resistance in adult *D. simulans* than exposure to each of the two stress factors alone. We observed this in all three experiments when testing our flies for desiccation resistance and in two of three experiments when testing them for heat resistance. The only exception was for heat hardening at 35°C. Although heat resistance increased here as compared with the non-hardened control both under low and high humidity conditions, it did not differ between the two treatments (35LH=35HH>control). Such a pattern may indicate that the plastic response resulting in improved thermotolerance is determined by a heat shock. Taking into account the data on Hsp70, one can also suggest that this response is associated with enhanced Hsp70 expression. The flies hardened at 35°C showed a markedly higher Hsp70 level than those stressed at lower temperatures irrespective of humidity conditions, and there was no difference in the amount of Hsp70 between 35LH and 35HH. Similarly, Krebs (Krebs, 1999) revealed in *D. simulans* at 35°C a positive effect of hardening on heat resistance as well as an increased level of Hsp70 expression. However, he found no hardening effect at a temperature of 36°C, which maximized the Hsp70 level, and concluded that large quantities of this Hsp in advance of stress are not required to achieve high thermotolerance.

In contrast to 35°C, the 31°C treatments improved heat resistance only in combination with low RH: the corresponding flies had higher knockdown time than those exposed to high RH and the control flies (31LH>31HH=control). The absence of a difference between 31HH and its control suggests that a temperature of 31°C does not change heat resistance substantially without desiccation stress, at least during the relatively short treatment time used in our experiment. This temperature is at the upper border of the normal temperature range for *D. simulans* (Cohet et al., 1980; David et al., 2004) and thus can be considered as mildly stressful. However, Krebs (Krebs, 1999) previously reported that heat resistance in *D. simulans* was increased after a preliminary exposure to 31°C under high humidity conditions. It is possible that there is some variation in this type of plastic response between different populations and laboratory strains of *D. simulans*.

We detected a higher Hsp70 concentration in all 31°C treatments as compared with the non-stressed control. At this temperature, the

Table 3. Comparisons between treatments within each experiment for four traits based on results of the Mann–Whitney *U*-test

Experiment	Treatment	Heat resistance	Desiccation resistance	Hsp70 expression level	Longevity
1	35LH	=35HH; >control***	>35HH***; >control***	=35HH; >control*	=35HH; <control***
	35HH	=35LH; >control***	<35LH***; =control	=35LH; >control*	=35LH; <control***
	Control	<35LH***; <35HH***	<35LH***; =35HH	<35LH*; <35HH*	>35LH***; >35HH***
2	31LH	>31HH*; >control**	>31HH***; >control***	>31HH*; >control*	
	31HH	<31LH*; =control	<31LH***; =control	<31LH*; >control*	
	Control	<31LH**; =31HH	<31LH***; =31HH	<31LH*; <31HH*	
3	31LH	>18LH*; >control***	>18LH***; >control***	>18LH*; >control*	=18LH; <control***
	18LH	<31LH*; >control*	<31LH***; =control	<31LH*; =control	=31LH; <control***
	Control	<31LH***; <18LH*	<31LH***; =18LH	<31LH*; =18LH	>31LH***; >18LH***

For treatment abbreviations, see Table 1.
Mathematical symbols indicate statistically significant differences (< or >) or their absence (=). *P<0.05, **P<0.01, ***P<0.001 (after the sequential Bonferroni correction).

Hsp70 level was higher at low than at high RH (31LH>31HH), indicating an enhanced Hsp70 synthesis under combined heat and desiccation stress. Such an effect has not been found in earlier *Drosophila* studies. At the same time, the results of heat resistance assays in the second experiment suggest that the heat-induced stress response associated with Hsp70 expression does not alone explain the pattern of heat resistance. However, the absence of a difference in Hsp70 level between 18LH and its control (third experiment) implies that Hsp70 level is not altered by low humidity at 18°C. This is in agreement with data by Sinclair et al. (Sinclair et al., 2007), who did not find increased Hsp70 expression in response to desiccation at room temperature in *D. melanogaster*. Thus, the situation with Hsp70 expression following only desiccation stress seems to be different in different insects. In contrast to *Drosophila*, such an expression has been previously reported for the flesh fly (Tammariello et al., 1999; Hayward et al., 2004), the bed bug (Benoit et al., 2009), the Antarctic midge (Lopez-Martinez et al., 2009) and some mosquito species (Benoit et al., 2010).

Whereas the exposure to 31°C in our study did not affect tolerance to heat without desiccation stress, low RH even at relatively low temperature resulted in increased heat resistance. This was observed in the third experiment, where the flies had a longer knockdown time at 31°C than at 18°C, but flies at the latter temperature were more heat resistant than the controls (31LH>18LH>control). The improved heat resistance after desiccation stress at 18°C indicates a cross-protection effect of desiccation hardening. Cross-protection effects appear as increased tolerance to some stress or a range of stresses after a treatment by a different stressor. In *D. melanogaster*, these effects were recently investigated by Bubliy et al. (Bubliy et al., 2012a), who found that acclimation and/or hardening to one stress commonly tends to decrease tolerance to other stresses. However, in the above-mentioned study, a desiccation hardening at a temperature of 21–23°C resulted in increased heat resistance, in agreement with our present results. The fact that tolerance to heat in 18LH was positively affected by desiccation hardening without changes in the Hsp70 level suggests that underlying physiological mechanisms are not explained by induction of this Hsp.

The desiccation resistance tests demonstrated increased tolerance to water loss after exposure to low humidity in combination with both 35 and 31°C (35LH>35HH=control, 31LH>31HH=control, 31LH>18LH=control), but no hardening effect was detected for low RH at 18°C. Apparently, the level of desiccation stress at 18°C was not high enough to induce a protective plastic response. It is known that drying power of the air, which is measured as vapour pressure deficit (VPD), rises rapidly with temperature. For RH of 20%, the VPD value is substantially (more than two times) higher at 31 than at 18°C. Thus, our case may illustrate the advantage of VPD over RH as a formal index of desiccation stress when different temperatures are compared. Previously, Hoffmann (Hoffmann, 1991) found that desiccation resistance in adult *D. simulans* was enhanced by low humidity hardening for 3, 4, 5 and 6 h at a relatively low temperature, being gradually increased with treatment duration. However, all of the treatments were performed at lower RH values than in our study and at a higher temperature of 25°C, which could provide more stressful conditions.

Finally, results from the desiccation resistance assays did not show increased tolerance to low humidity conditions after prior exposures to heat at high RH, i.e. unlike desiccation hardening, the applied heat stress did not reveal a cross-protection effect. Bubliy et al. (Bubliy et al., 2012a) found that heat hardening at 36°C and high RH in adult *D. melanogaster* even reduced desiccation resistance as compared with the non-hardened controls. In contrast, Hoffmann (Hoffmann, 1990) observed an increase in desiccation tolerance for this species after a heat shock at 35°C. However, the humidity level was not reported in his paper and it is possible that the flies were also exposed to some desiccation stress.

The physiological mechanisms underlying the plastic response to desiccation hardening in *Drosophila* remain unclear. Though dehydration has been shown to boost Hsp70 expression in some insects (see references above) and the same probably occurred at 31°C in our second experiment, a close association between Hsp70 and desiccation resistance seems unlikely: we did not find changes in desiccation resistance in the flies with increased Hsp70 levels exposed to high humidity (35HH and 31HH). The pattern of cross-resistance in our previous study (Bubliy et al., 2012a) indirectly indicated that a reduced metabolism might be responsible for increased desiccation tolerance in *D. melanogaster* after hardening at low RH. It is known that terrestrial animals lose water in the process of respiration (Schmidt-Nielsen, 1997), and some recent findings demonstrate a strong correlation between the water loss rate and the rate of exchange for metabolic gases (Woods and Smith, 2010). However, the contribution of respiratory transpiration to overall water loss is not always evident in insect studies, including those performed on *Drosophila* (for reviews, see Chown, 2002; Chown and Nicolson, 2004). For *D. melanogaster*, Bazinet et al. (Bazinet et al., 2010) reported that a desiccation pre-treatment did not affect metabolic rate, and the reduction in water loss rate found in their experiment was most likely based on a change in cuticular permeability. There is evidence that *Drosophila* may respond to low humidity treatments using other protective mechanisms. For example, Parkash et al. (Parkash et al., 2012a) detected an increased level of carbohydrates, particularly trehalose, after a successful

hardening to dehydration stress in *D. immigrans*. Trehalose is known to be one of the most commonly stored sugars in insects (Chapman, 1998) and it might bind extra water, thereby restricting its loss under low humidity conditions.

In the context of adaptation to survival in unfavourable environments, the observed plastic responses induced by combined heat and desiccation stress can be viewed as beneficial for *D. simulans*. Because this cosmopolitan species occurs in a wide range of habitats, such stress seems to be common, particularly in regions with dry and hot climates. The effect of combined hardening might be even more important for cactophilic desert species of *Drosophila*, which can be exposed to extremely high diurnal temperatures and low humidities both inside and outside cactus rots (Gibbs et al., 2003). It is probably no coincidence that the most heat-resistant species in this genus were found among desert endemics (Stratman and Markow, 1998). A large-scale among-species comparison performed by Kellerman et al. (Kellerman et al., 2013) demonstrated a strong association between heat and desiccation resistance in *Drosophila* from hot and dry climates, suggesting correlated selection for these traits under combined heat and desiccation stress. Recent data also indicate a significant role of desiccation stress in thermal adaptation of *Drosophila* across different climates. Kellermann et al. (Kellermann et al., 2012) found a weak positive correlation between heat resistance of species and average as well as maximal temperature at the central point of their distribution. In contrast, there was a rather strong negative correlation with annual precipitation, implying that factors related to water in the environment are more important in determining the distribution of species than high temperature alone. At the intraspecific level, evidence on importance of desiccation stress for evolutionary adaptation to high temperatures was obtained by Bubliy et al. (Bubliy et al., 2012b), who investigated the effect of extremely low humidity on genetic variation for heat resistance in *D. melanogaster*.

In our longevity assays, we revealed that exposures to combined temperature and desiccation stress as well as treatments using only one of these stress factors can negatively affect fitness. Both highly and mildly stressed flies (35LH, 35HH, 31LH, 18LH) had a reduced life span under non-stressful conditions compared with their controls. At the same time, no difference in longevity was found between the hardening treatments within experiments (35LH=35HH, 31LH=18LH). Taking into account the mortality values, it is reasonable to suggest that some individuals with low fitness and potentially shorter life span might die immediately after the highly stressful treatments. In this case, the corresponding longevity estimates might be slightly inflated. Our results are in agreement with those of Bubliy et al. (Bubliy et al., 2012a), who found a decrease in life span for heat- and desiccation-hardened *D. melanogaster* under non-stressful conditions. However, there is evidence of beneficial (hormetic) effects of high temperature treatments on longevity in *Drosophila* (Khazaeli et al., 1997; Le Bourg et al., 2001; Hercus et al., 2003; Scannapieco et al., 2007). It is likely that the stress doses applied in our experiments to maximize the beneficial effect of treatments on stress resistance could be larger than those used by the above-mentioned authors.

From an evolutionary point of view, the cost and benefit of the plastic response to hardening and acclimation is covered by a trade-off between increased resistance, enabling immediate survival, and reduced reproduction and longevity. The negative effects on fitness imply that in stressful environments selection for such plastic response is constrained and genotypes with increased basal tolerance levels may be preferred. Genes responsible for costly plastic changes should not be favoured by natural selection in populations that are never or rarely exposed to extreme conditions. It has been suggested (see Hoffmann and Parsons, 1991) that the highest levels of phenotypic plasticity for physiological traits occur in species and populations from stressful fluctuating environments. Because many widespread *Drosophila* species, including *D. simulans*, occupy a range of different habitats, one might expect interpopulation variation in plastic responses to environmental stresses. However, evidence on such variation for heat and desiccation resistance traits in *Drosophila* is still scarce and sometimes controversial (Levins 1969; Hoffmann 1991; Hoffmann and Watson 1993; Parkash et al., 2012a).

It is apparent that more extensive population studies as well as among-species comparisons are needed for a better understanding of the evolution of the above-mentioned physiological plastic responses in *Drosophila*. Laboratory experiments, where possible, should be complemented with field observations to take into account various factors affecting fitness under natural conditions. It is known, for instance, that adult *Drosophila* try to avoid uncomfortably thermal extremes by micro-habitat selection (Junge-Berberovic, 1996; Feder et al., 2000), which is a kind of behavioural adaptation. Though currently there is no doubt that stress-induced physiological responses contribute to adaptation of insects to extreme environments, their importance may be somewhat overestimated. Future studies should shed light on this issue.

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AUTHOR CONTRIBUTIONS

O.A.B., T.N.K. and V.L. designed the research; O.A.B. performed the research and analyzed the data; and O.A.B., T.N.K. and V.L. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

Addo-Bediako, A., Chown, S. L. and Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proc. Biol. Sci.* **267**, 739-745.

Aggarwal, D. D., Ranga, P., Kalra, B., Parkash, R., Rashkovetsky, E. and Bantis, L. E. (2013). Rapid effects of humidity acclimation on stress resistance in *Drosophila melanogaster*. *Comp. Biochem. Physiol.* **166A**, 81-90.

Bazinet, A. L., Marshall, K. E., MacMillan, H. A., Williams, C. M. and Sinclair, B. J. (2010). Rapid changes in desiccation resistance in *Drosophila melanogaster* are facilitated by changes in cuticular permeability. *J. Insect Physiol.* **56**, 2006-2012.

Benoit, J. B., Lopez-Martinez, G., Teets, N. M., Phillips, S. A. and Denlinger, D. L. (2009). Responses of the bed bug, *Cimex lectularius*, to temperature extremes and dehydration: levels of tolerance, rapid cold hardening and expression of heat shock proteins. *Med. Vet. Entomol.* **23**, 418-425.

Benoit, J. B., Lopez-Martinez, G., Phillips, Z. P., Patrick, K. R. and Denlinger, D. L. (2010). Heat shock proteins contribute to mosquito dehydration tolerance. *J. Insect Physiol.* **56**, 151-156.

Bowler, K. (2005). Acclimation, heat shock and hardening. *J. Therm. Biol.* **30**, 125-130.

Bowler, K. and Terblanche, J. S. (2008). Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? *Biol. Rev. Camb. Philos. Soc.* **83**, 339-355.

Bubliy, O. A., Kristensen, T. N., Kellermann, V. and Loeschcke, V. (2012a). Plastic responses to four environmental stresses and cross-resistance in a laboratory population of *Drosophila melanogaster*. *Funct. Ecol.* **26**, 245-253.

Bubliy, O. A., Kristensen, T. N., Kellermann, V. and Loeschcke, V. (2012b). Humidity affects genetic architecture of heat resistance in *Drosophila melanogaster*. *J. Evol. Biol.* **25**, 1180-1188.

Chapman, R. F. (1998). *The Insects: Structure and Function*. Cambridge: Cambridge University Press.

Chown, S. L. (2002). Respiratory water loss in insects. *Comp. Biochem. Physiol.* **133A**, 791-804.

Chown, S. L. and Nicolson, S. W. (2004). *Insect Physiological Ecology: Mechanisms and Patterns*. Oxford: Oxford University Press.
Chown, S. L. and Terblanche, J. S. (2006). Physiological diversity in insects: ecological and evolutionary contexts. *Adv. Insect. Physiol* **33**, 50-152.
Chown, S. L., Sørensen, J. G. and Terblanche, J. S. (2011). Water loss in insects: an environmental change perspective. *J. Insect Physiol.* **57**, 1070-1084.
Cohet, Y., Vouidibio, J. and David, J. R. (1980). Thermal tolerance and geographic distribution: a comparison of cosmopolitan and tropical endemic *Drosophila* species. *J. Therm. Biol.* **5**, 69-74.
David, J. R., Allemand, R., Capy, P., Chakir, M., Gibert, P., Pétavy, G. and Moreteau, B. (2004). Comparative life histories and ecophysiology of *Drosophila melanogaster* and *D. simulans*. *Genetica* **120**, 151-163.
Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.
Feder, M. E. and Krebs, R. A. (1997). Ecological and evolutionary physiology of heat shock proteins and the stress response in *Drosophila*: complementary insights from genetic engineering and natural variation. In *Environmenta Stress, Adaptation, and Evolution* (ed. R. Bijlsma and V. Loeschcke), pp. 155-173. Basel: Birkhäuser Verlag.
Feder, M. E., Roberts, S. P. and Bordelon, A. C. (2000). Molecular thermal telemetry of free-ranging adult *Drosophila melanogaster*. *Oecologia* **123**, 460-465.
Gibbs, A. G. and Markow, T. A. (2001). Effects of age on water balance in *Drosophila* species. *Physiol. Biochem. Zool.* **74**, 520-530.
Gibbs, A. G., Perkins, M. C. and Markow, T. A. (2003). No place to hide: microclimates of Sonoran Desert *Drosophila*. *J. Therm. Biol.* **28**, 353-362.
Hayward, S. A. L., Rinehart, J. P. and Denlinger, D. L. (2004). Desiccation and rehydration elicit distinct heat shock protein transcript responses in flesh fly pupae. *J. Exp. Biol.* **207**, 963-971.
Hercus, M. J., Loeschcke, V. and Rattan, S. I. S. (2003). Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress. *Biogerontology* **4**, 149-156.
Hoffmann, A. A. (1990). Acclimation for desiccation resistance in *Drosophila melanogaster* and the association between acclimation responses and genetic variation. *J. Insect Physiol.* **36**, 885-891.
Hoffmann, A. A. (1991). Acclimation for desiccation resistance in *Drosophila*: species and population comparisons. *J. Insect Physiol.* **37**, 757-762.
Hoffmann, A. A. and Blows, M. W. (1994). Species borders: ecological and evolutionary perspectives. *Trends Ecol. Evol.* **9**, 223-227.
Hoffmann, A. A. and Parsons, P. A. (1991). *Evolutionary Genetics and Environmental Stress*. Oxford: Oxford University Press.
Hoffmann, A. A. and Watson, M. (1993). Geographical variation in the acclimation responses of *Drosophila* to temperature extremes. *Am. Nat.* **142 Suppl. 1**, S93-S113.
Hoffmann, A. A., Sørensen, J. G. and Loeschcke, V. (2003). Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *J. Therm. Biol.* **28**, 175-216.
Junge-Berberovic, R. (1996). Effect of thermal environment on life histories of free living *Drosophila melanogaster* and *D. subobscura*. *Oecologia* **108**, 262-272.
Kellermann, V., Overgaard, J., Hoffmann, A. A., Fløjgaard, C., Svenning, J. C. and Loeschcke, V. (2012). Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proc. Natl. Acad. Sci. USA* **109**, 16228-16233.
Kellermann, V., Overgaard, J., Loeschcke, V., Kristensen, T. N. and Hoffmann, A. A. (2013). Trait associations across evolutionary time within a *Drosophila* phylogeny: correlated selection or genetic constraint? *PLoS ONE* **8**, e72072.
Khazaeli, A. A., Tatar, M., Pletcher, S. D. and Curtsinger, J. W. (1997). Heat-induced longevity extension in *Drosophila*. I. Heat treatment, mortality, and thermotolerance. *J. Gerontol.* **52A**, B48-B52.
Krebs, R. A. (1999). A comparison of Hsp70 expression and thermotolerance in adults and larvae of three *Drosophila* species. *Cell Stress Chaperones* **4**, 243-249.
Krebs, R. A. and Loeschcke, V. (1994). Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. *Funct. Ecol.* **8**, 730-737.
Lachaise, D. and Silvain, J. F. (2004). How two Afrotropical endemics made two cosmopolitan human commensals: the *Drosophila melanogaster–D. simulans* palaeogeographic riddle. *Genetica* **120**, 17-39.
Le Bourg, E., Valenti, P., Lucchetta, P. and Payre, F. (2001). Effects of mild heat shocks at young age on aging and longevity in *Drosophila melanogaster*. *Biogerontology* **2**, 155-164.
Levins, R. (1969). Thermal acclimation and heat resistance in *Drosophila* species. *Am. Nat.* **103**, 483-499.
Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* **55**, 1151-1191.
Lopez-Martinez, G., Benoit, J. B., Rinehart, J. P., Elnitsky, M. A., Lee, R. E., Jr and Denlinger, D. L. (2009). Dehydration, rehydration, and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *J. Comp. Physiol. B* **179**, 481-491.
Parkash, R., Aggarwal, D. D., Ranga, P. and Singh, D. (2012a). Divergent strategies for adaptation to desiccation stress in two *Drosophila* species of immigrans group. *J. Comp. Physiol. B* **182**, 751-769.
Parkash, R., Ramniwas, S., Kajla, B. and Aggarwal, D. D. (2012b). Divergence of desiccation-related traits in two *Drosophila* species of the *takahashii* subgroup from the western Himalayas. *J. Exp. Biol.* **215**, 2181-2191.
Rice, W. R. (1989). Analyzing tables of statistical tests. *Evolution* **43**, 223-225.
Scannapieco, A. C., Sørensen, J. G., Loeschcke, V. and Norry, F. M. (2007). Heat-induced hormesis in longevity of two sibling *Drosophila* species. *Biogerontology* **8**, 315-325.
Schmidt-Nielsen, K. (1997). *Animal Physiology: Adaptation and Environment*, 5th edn. Cambridge: Cambridge University Press.
Sinclair, B. J., Vernon, P., Klok, C. J. and Chown, S. L. (2003). Insects at low temperatures: an ecological perspective. *Trends Ecol. Evol.* **18**, 257-262.
Sinclair, B. J., Gibbs, A. G. and Roberts, S. P. (2007). Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect Mol. Biol.* **16**, 435-443.
Solomon, J. M., Rossi, J. M., Golic, K., McGarry, T. and Lindquist, S. (1991). Changes in hsp70 alter thermotolerance and heat-shock regulation in *Drosophila*. *New Biol.* **3**, 1106-1120.
Sørensen, J. G. and Loeschcke, V. (2002). Decreased heat-shock resistance and down-regulation of Hsp70 expression with increasing age in adult *Drosophila melanogaster*. *Funct. Ecol.* **16**, 379-384.
Sørensen, J. G., Michalak, P., Justesen, J. and Loeschcke, V. (1999). Expression of the heat-shock protein HSP70 in *Drosophila buzzatii* lines selected for thermal resistance. *Hereditas* **131**, 155-164.
Stratman, R. and Markow, T. A. (1998). Resistance to thermal stress in desert *Drosophila*. *Funct. Ecol.* **12**, 965-970.
Tammariello, S. P., Rinehart, J. P. and Denlinger, D. L. (1999). Desiccation elicits heat shock protein transcription in the flesh fly, *Sarcophaga crassipalpis*, but does not enhance tolerance to high or low temperatures. *J. Insect Physiol.* **45**, 933-938.
Tatar, M. (1999). Transgenes in the analysis of life span and fitness. *Am. Nat.* **154**, S67-S81.
Velazquez, J. M. and Lindquist, S. (1984). hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. *Cell* **36**, 655-662.
Welte, M. A., Tetrault, J. M., Dellavalle, R. P. and Lindquist, S. L. (1993). A new method for manipulating transgenes: engineering heat tolerance in a complex, multicellular organism. *Curr. Biol.* **3**, 842-853.
Woods, H. A. and Smith, J. N. (2010). Universal model for water costs of gas exchange by animals and plants. *Proc. Natl. Acad. Sci. USA* **107**, 8469-8474.