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RESEARCH ARTICLE

Small heat shock protein Hsp67Bc plays a significant role in *Drosophila melanogaster* cold stress tolerance

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ABSTRACT

Hsp67Bc in *Drosophila melanogaster* is a member of the small heat shock protein family, the main function of which is to prevent the aggregation of misfolded or damaged proteins. Hsp67Bc interacts with Starvin and Hsp23, which are known to be a part of the cold stress response in the fly during the recovery phase. In this study, we investigated the role of the *Hsp67Bc* gene in the cold stress response. We showed that in adult *Drosophila*, *Hsp67Bc* expression increases after cold stress and decreases after 1.5 h of recovery, indicating the involvement of Hsp67Bc in short-term stress recovery. We also implemented a deletion in the *D. melanogaster Hsp67Bc* gene using imprecise excision of a *P*-element, and analysed the cold tolerance of *Hsp67Bc*-null mutants at different developmental stages. We found that *Hsp67Bc*-null homozygous flies are viable and fertile but display varying cold stress tolerance throughout the stages of ontogenesis: the survival after cold stress is slightly impaired in late third instar larvae, unaffected in pupae, and notably affected in adult females. Moreover, the recovery from chill coma is delayed in *Hsp67Bc*-null adults of both sexes. In addition, the deletion in the *Hsp67Bc* gene caused more prominent up-regulation of *Hsp70* following cold stress, suggesting the involvement of *Hsp70* in compensation of the lack of the Hsp67Bc protein. Taken together, our results suggest that Hsp67Bc is involved in the recovery of flies from a comatose state and contributes to the protection of the fruit fly from cold stress.

KEY WORDS: Cold stress, *Drosophila*, Hsp67Bc, Small heat shock proteins

INTRODUCTION

Drosophila species are ectothermic animals, and plasticity in thermal tolerance is crucial to them. Resistance to cold defines fruit fly activity, reproductive success, and as a consequence, their habitat. In the laboratory, cold tolerance is generally measured in one of the following ways: acute cold stress (0°C, up to 3 h), prolonged (chronic) cold stress (0°C, 6 h or more), and long-term exposure at low temperature (+5 to +15°C, days or weeks) (Rajamohan and Sinclair, 2008; Sinclair and Roberts, 2005). Short cooling to non-lethal low temperatures causes rapid cold hardening and increases the survival of flies upon repeated exposure to cold (Overgaard et al., 2005; Vesala et al., 2012). Acute cold stress is assumed to cause cell membrane depolarization, primarily in muscle cells and neurons, thereby leading to paralysis (Andersen and Overgaard, 2019). Longer-term or sustained cold exposures differ from acute stress in the nature of the caused injury: prolonged cell membrane depolarization results in a disruption of ion and water homeostasis, thus causing more severe damage to the cell (Overgaard and MacMillan, 2017; Sinclair and Roberts, 2005). As an example, in insects, chronic cold stress results in an increase of K^+ concentration in hemolymph, which, in combination with sub-optimal cooling, may lead to cell death (Overgaard and MacMillan, 2017). Long-term exposure to mild low temperature results in cold acclimation and increases resistance to cold in almost all insects, including *Drosophila*, as extensive transcriptomic and metabolomic shifts occur in acclimated individuals (MacMillan et al., 2016). Chill-tolerant insects have an improved ability to maintain homeostatic balance at low temperatures due to higher hemolymph osmolality, accumulation of cryoprotective osmolytes, and modification of cell membrane phospholipid composition (Overgaard and MacMillan, 2017).

A common method for investigating cold tolerance in *Drosophila* is chill coma: a state of complete neuromuscular paralysis reached by individuals at the critical thermal minimum. The immobilization is reversible because after being transferred to an environment of permissive temperature, the flies recover and stand back up. This method was first suggested by David et al. (1998) and subsequently has become widespread (e.g. Andersen and Overgaard, 2019; Colinet et al. 2010a,b,c). Chill coma onset temperature and chill coma recovery time vary across fly populations and can be used as a measure of fly cold tolerance (Andersen and Overgaard, 2019; David et al., 1998; Macdonald et al., 2004; Vesala et al., 2012). To date, chill coma onset temperature and chill coma recovery time have strictly correlated with latitudinal distribution of fly populations (Andersen and Overgaard, 2019).

Research has revealed an expression change in a great variety of genes in response to cold in *Drosophila melanogaster* (Qin et al., 2005; Zhang et al., 2011), with up to a third of the genome differentially regulated after prolonged cold exposure (MacMillan et al., 2016; von Heckel et al., 2016). In all instances, the most prominent transcriptional response to all the types of cold treatment was found in genes coding for heat shock proteins (HSPs) (MacMillan et al., 2016; Qin et al., 2005; von Heckel et al., 2016; Zhang et al., 2011). HSPs are present in all living organisms and are crucial for coping with various stressors, including high and low temperatures (Colinet et al., 2010c; Lindquist, 1986). Studies on other *Drosophila* species, namely *D. ananassae*, *D. montana* and *D. virilis*, have shown cold-induced expression of genes *Hsp22*, *Hsp23*, *Hsp26*, *Hsp27*, *Hsp40*, *Hsp67Ba*, *Hsp67Bc*, *Hsp68*, *Hsp83* and *Hsp70* (Königer and Grath, 2018; Parker et al., 2015; Vesala et al., 2012). More than half of these genes belong to the small heat shock protein (sHsp) family of HSPs.

The highly conserved sHsp family is characterized by the presence of an α-crystallin domain. The major function of sHsps in the cell is the prevention of aggregation of misfolded and damaged proteins, and sHsps are reported to be some of the most up-regulated

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HSPs following stress (Morrow and Tanguay, 2015). In *D. melanogaster*, 12 sHsp genes have been identified: *CG4461*, *CG7409*, *CG13133*, *CG14207*, *CG43851*, *Hsp22*, *Hsp23*, *Hsp26*, *Hsp27*, *Hsp67Ba*, *Hsp67Bc* and *l(2)efl* (Morrow and Tanguay, 2015). Nonetheless, only four sHsp proteins (Hsp22, Hsp23, Hsp26 and Hsp27) have been thoroughly investigated (Colinet et al., 2010c; Morrow and Tanguay, 2015). Some sHsp genes are known to be up-regulated during recovery from cold temperatures (Colinet et al., 2010a; von Heckel et al., 2016). Recently, it was demonstrated that another sHsp family member, the *Hsp67Bc* gene, is up-regulated in cold-acclimated flies (MacMillan et al., 2016).

In *D. melanogaster*, the Hsp67Bc protein has been shown to be the functional orthologue of human HSPB8 and to interact with Starvin (Stv) (Carra et al., 2010). Stv is essential for viability and muscle maintenance and is enriched at Z-discs in muscle tissue, where both HSPB8 and Hsp67Bc can be found (Arndt et al., 2010; Carra et al., 2010; Coulson et al., 2005). *Stv* expression was also demonstrated to be up-regulated during the recovery phase in *Drosophila* alongside *Hsp70* expression (Colinet and Hoffmann, 2010). *Hsp67Bc* gene expression has been reported to increase in *Drosophila* embryos and S2 cells following heat stress (Leemans et al., 2000; Vos et al., 2016) and in cold-acclimated adult flies (MacMillan et al., 2016). Here, we describe a newly obtained *D. melanogaster* line with a deletion in the *Hsp67Bc* gene and investigate the effects of cold stress on these mutant flies. We found that *Hsp67Bc*, alongside other *sHsp* genes, participates in cold tolerance in *D. melanogaster*.

MATERIALS AND METHODS

Drosophila melanogaster lines and genotypes

All the *Drosophila melanogaster* Meigen 1830 flies were raised at 24–25°C and ∼60% relative humidity on a standard cornmeal–agar medium. All the lines initially infected by endosymbiotic *Wolbachia* bacteria were treated with 0.42 mg ml^{-1} doxycycline (i.e. kept on antibiotic-containing food for one generation). After that, all the fly lines were found to be PCR-negative for *Wolbachia* infection. The offspring were then raised on standard food.

The initial *D. melanogaster* lines included: (i) w^{1118} whose DNA served as a control in classic PCR (kindly provided by Professor Scott O'Neill, University of Queensland, Australia); (ii) d06009 (genotype *P{XP}Hsp67Bc*^{d06009}), with the insertion of a *mini-white* gene-coding *P*-element {XP} of 7303 bp (GenBank: AY515149.1) 115 bp upstream of the *Hsp67Bc* gene on the third chromosome (Exelixis at Harvard Medical School); and (iii) transposase-coding line *y w; Ki, P{Δ2-3}99B* from laboratory stock.

The *D. melanogaster* line with a deletion in the *Hsp67Bc* gene was obtained by the method of imprecise excision of a *P*-element. For this purpose, the d06009 line was crossed to the *y w; Ki, P{Δ2-3}99B* line. The detailed scheme of the crosses is presented in Fig. 1. F1 males, all having both the *P*-element and transposase, were crossed to previously obtained *D. melanogaster* females with one of the third chromosomes coding *P{XP}Hsp67Bc*^{d06009} and the other being a balancer (*w; P{XP}Hsp67Bc*^{d06009}*/TM6B, Hu*). From F2, males with white eyes (meaning that the *P*-element was cut out and lost) were selected and back-crossed to *w; P{XP}Hsp67Bc*^{d06009}*/TM6B, Hu* females to subsequently obtain a stable line. After that, the DNA of the F2 males was extracted and analysed by PCR to detect a desired genotype variant. Such a fly line should have a deletion affecting only the *Hsp67Bc* gene (the amplicon of a region including only *Hsp67Bc* should be shorter than that of the control w^{1118} line) and no remains of the *P*-element. Of the 278 obtained lines, only one matched these criteria, while the others either had a normal-size amplicon (majority), meaning the precise excision of the *P*-element, or no amplicon at all, meaning that either the deletion affected an area outside the *Hsp67Bc* gene or that there was still a part of the *P*-element (without the *mini-white* gene) remaining. In the obtained appropriate line, the part of the third chromosome containing the *Hsp67Bc* gene and *P*-element {XP} insertion site was then sequenced. As part of the experiment, a line from which the *P*-element was cut out precisely was chosen (based on DNA sequence similarities with reference and mutant ones) and used as a control.

Classic PCR

To find a mutant variant with the deletion affecting only the *Hsp67Bc* gene among the obtained *D. melanogaster* lines, classic PCR was carried out. From each line, one male was homogenized in STE buffer (100 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl, pH 8.0,

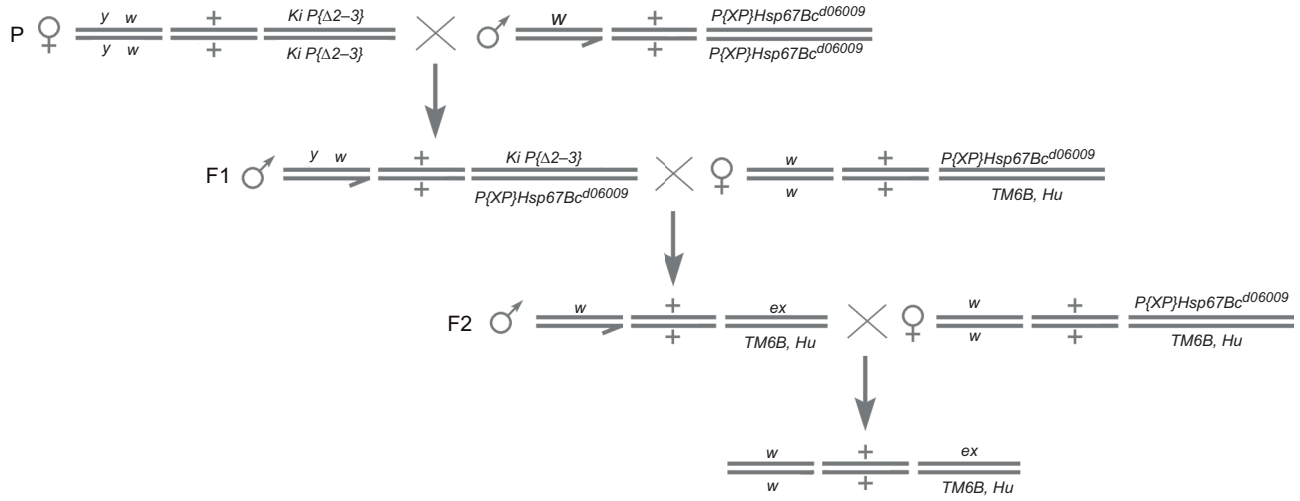

Fig. 1. The scheme of crosses resulting in a *Drosophila melanogaster* line with a deletion in the *Hsp67Bc* gene. *y w; Ki, P{Δ2-3}99B* females carrying a transposase gene were crossed to *P{XP}Hsp67Bc*^{d06009} males carrying a *P*-element 115 bp upstream of the *Hsp67Bc* gene's start codon (strain d06009). F1 males were then crossed to females with *P{XP}Hsp67Bc*^{d06009} balanced by *TM6B, Hu*. F2 males with white eyes (meaning that the *P*-element was cut out and lost) were selected and back-crossed to *w; P{XP}Hsp67Bc*^{d06009}*/TM6B, Hu* females to subsequently obtain a stable line with the *P*-element excision (*ex*).

and 1 mmol l^{-1} EDTA) with SDS and proteinase K at final concentrations of 1% and 0.2 mg ml^{-1}, respectively. The homogenate was then incubated at 56°C for 90 min, followed by 8 min at 95°C and brief centrifugation. Next, the DNA was precipitated with 0.5 volumes of 5 mol l^{-1} sodium acetate and 2 volumes of absolute ethanol, followed by washing in 75% ethanol and dissolution in deionized water. Twenty-microlitre PCRs based on the BioMaster HS-Taq PCR-Color (2×) mix (Biolabmix, Novosibirsk, Russia) were set up with primers complementary to regions 285 bp upstream (left primer: 5′-GATTGCGCCTCTCAT-CTGTATC-3′) and 236 bp downstream (right primer: 5′-ATTAC-ACTTATGGCGGGCTTTC-3′) of *Hsp67Bc*. The thermal cycling protocol for the amplification was as follows: 5 min denaturation at 95°C; followed by 30 cycles of 30 s at 95°C, 30 s at 57°C, and 45 s at 72°C; and finally, 5 min of elongation at 72°C. The PCR products were separated by electrophoresis on a 1% agarose gel in TBE buffer.

The primers were chosen based on the reverse complement of GenBank sequence AE014296.5.

DNA sequencing

To characterize the obtained *Hsp67Bc*-null fly line, sequencing of the mutant DNA fragment containing the *Hsp67Bc* gene was performed. DNA was extracted from 2–3 flies and amplified as described in the 'Classic PCR' section above. DNA quality was checked by electrophoresis. The amplified DNA was precipitated with 20% polyethylene glycol and 75% ethanol and dissolved in deionized water. The sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) and with the same primers as those employed for the classic PCR. The cycle sequencing program was as follows: incubation at 95°C for 1 min followed by 35 cycles of 10 s at 98°C, 10 s at 50°C, and 4 min at 60°C. The sequencing reaction mixture was then purified with 7.5 mol l^{-1} sodium acetate and absolute ethanol, followed by washing in 75% ethanol, after which it was dried and handed over for sequencing to the Genomics Core Facility (Institute of Chemical Biology and Fundamental Medicine, SB RAS, Novosibirsk, Russia). The results were then analysed in the BioEdit Sequence Alignment Editor software, version 7.2.5 (https://bioedit.software.informer.com/7.2/).

Cold stress applied to Hsp67Bc-0 larvae and pupae

For these experiments, wandering late third instar (L3) larvae were transferred from the walls of their rearing vials to the walls of vials with fresh food, at 20 per tube (30 in a few early tests). The larvae were then allowed to reach a developmental stage that was to be treated, in particular, immobile late L3, white prepupa (less than 1 h after pupation (Bainbridge and Bownes, 1981), with slight addition of early P2 stage prepupae with yellow puparium), P4–P5 (11–13 h after pupation), or P7–P8 (46–48 h after pupation), which correspond to the highest *Hsp67Bc* expression levels according to FlyBase (http://flybase.org/). The vials containing *D. melanogaster* individuals were then transferred to a 0°C environment (ice bath) for 2, 4 or 12 h incubation. After each treatment, the flies were kept at 24–25°C until eclosion. Survivors to the pupa stage (in case of late L3 larvae treatment) and to the adult stage (for all treatment groups) were then counted, and the resultant number was divided by the initial number of individuals in the group to assess the survival rates. Each experiment was repeated 4–16 times.

Chill coma recovery and survival of adult *Drosophila*

Three-day-old *D. melanogaster* individuals were sorted by sex and placed into vials at 15 flies per vial, one day prior to the start of the experiment. The chill coma assay was based on a method described by Colinet et al. (2010c): the vials with 4-day-old flies were placed in a 0° C (ice bath) environment for 2, 4 or 12 h incubation, after which they were transferred to a 24–25°C environment to recover. The recovery was then measured by counting the flies that were standing, every 5 min (2 and 4 h chill coma) or 10 min (12 h chill coma) after the end of the cold stress. For this experiment, three (2 and 4 h chill coma) or seven (12 h chill coma) iterations with 60–107 flies in each were carried out. Recovery curves were constructed based on averaged results. For the statistical analyses, separate experiments were compared. Two days (48 h) after the cold treatment, the surviving flies were counted. Survival rates of the flies were calculated as a ratio of the surviving individuals to the initial number of individuals.

Fecundity assays

One day prior to the start of the experiment, 3-day-old *D. melanogaster* individuals were placed into vials at either eight males and 10 females per each of 10 vials in the experimental group, or six males and eight females per each of four vials in the control group. The control group was kept at 24.5°C; the vials with 4-day-old individuals from the experimental group were placed in a 0°C (ice bath) environment for 12 h incubation, after which they were transferred to a 24.5°C environment. The fecundity of *D. melanogaster* in both groups was measured every day for one week, starting one day after the end of the exposure to cold. The females were allowed to lay eggs for 24 h in vials with fresh food, then they were transferred to new food, and the eggs were counted under a binocular microscope. The quantity of eggs in each vial was then divided by the number of females that oviposited in that very vial.

Increasing the initial number of individuals in vials, as well as number of the vials, in the experimental group compared with the control group allowed us to have similar sample sizes throughout most of the fecundity experiment because preliminary chill coma survival assays revealed that some females died during the first two days after the chill coma.

RNA extraction and complementary DNA synthesis

Total RNA was extracted from whole adults (three males or females per sample, or four larvae or pupae per sample) using the TRIzol Reagent analogue from Biolabmix (Novosibirsk, Russia) according to the instructions given in the Invitrogen protocol for the TRIzol Reagent. RNA concentrations were determined on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA contamination was then eliminated using DNase I with the addition of the RiboLock RNase Inhibitor (both from Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Complementary DNA was synthesized by means of the oligo$(dT)_{17}$ primer (Evrogen, Moscow, Russia) and RevertAid Reverse Transcriptase (Thermo Fisher Scientific).

Quantitative real-time PCR (qPCR)

This procedure was performed on a LightCycler 96 cycler (Roche). The thermal cycling protocol for the amplification reaction was as follows: 5 min pre-incubation at 95°C; next, 40 cycles of 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C; followed by a melting curve program at 65–97°C. The results were then analysed in REST 2009 software (https://www.gene-quantification.de/rest-2009.html).

Primers used in qPCR reactions are listed in Table 1. The primer sequences for *αTubulin84B* were taken from Ponton et al. (2011); for *Hsp22*, from Colinet et al. (2010c); and for *Fst*, from Newman et al. (2017). *αTubulin84B* was chosen as a reference gene based on reports of its stability across various temperature ranges (Myachina et al., 2017).

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Table 1. The list of primers used in qPCR

Gene	Primer	Sequence
αTubulin84B	Forward	5′-TGTCGCGTGTGAAACACTTC-3′
	Reverse	5′-AGCAGGCGTTTCCAATCTG-3′
Hsp67Bc	Forward	5′-GTCAAGGTGAATTGTGCGCCAAACT-3′
	Reverse	5′-AACCGACGACCTGTTTTCCT-3′
stv	Forward	5′-GACACAAACGGCAAGGACAG-3′
	Reverse	5′-CCGCTCTGGCATTCTCCTC-3′
Hsp67Ba	Forward	5′-ATGTCGCTGATACCGTTCATAC-3′
	Reverse	5′-CCAGTGGATACAACCCGAATC-3′
Hsp70	Forward	5′-ACCTCAACCTATCCATCAACCC-3′
	Reverse	5′-ATTACACCTCCAGCGGTCTC-3′
Hsp22	Forward	5′-GCCTCTCCTCGCCCTTTCAC-3′
	Reverse	5′-TCCTCGGTAGCGCCACACTC-3′
Hsp23	Forward	5′-TCACTTTGTCCGCCGCTATG-3′
	Reverse	5′-ATGCGCTCGTTGCCCTTATC-3′
l(2)efl	Forward	5′-AGGGACTGAAGAGGGATGAC-3′
	Reverse	5′-CACATCCAGAATGACCTCGAAC-3′
Fst	Forward	5′-AGTGGAATCCAAATGGCAAC-3′
	Reverse	5′-ATCCTCGGTGGTCAACTCAG-3′

Statistical analyses

The comparison of survival rates after cold treatment between lines was performed by the chi-squared test. Recovery curves were compared within each experiment by the Kolmogorov–Smirnov test, and the conclusion about significance of a difference in each experiment within the treatment groups was based on the majority of the test results. Differences between mutant and control lines in the fecundity of females that underwent the chill coma and at 24.5°C were compared by the heteroscedastic *t*-test at each time point throughout the experiment to reveal possible differences in fecundity dynamics. The procedure was followed by application of the Benjamini–Krieger–Yekutieli method to control the false discovery rate. To discover differences between the mutant and control fly lines in mean fecundity throughout the experiment, total averages from all the registration points were compared by the heteroscedastic *t*-test.

RESULTS

Implementation of the deletion in the *Hsp67Bc* gene

As a result of the imprecise excision of the *P*-element {XP} located 115 bp upstream of the *Hsp67Bc* gene in parent line d06009, a *D. melanogaster* line with a deletion of almost the whole gene was obtained. PCR analysis revealed that in this line, the length of the amplicon between primers complementary to regions 285 bp upstream and 236 bp downstream of the *Hsp67Bc* gene was approximately 450 bp, unlike 1508 bp in the control (Fig. 2A). Sequencing of DNA from the obtained mutant line revealed a deletion of 1076 bp including the *Hsp67Bc* promoter (position 9,371,643 to position 9,372,718 on chromosome 3L in GenBank sequence AE014296.5), leaving only the last 132 bp from the 3′-end of the 987 bp *Hsp67Bc* gene (Fig. 2B). In addition, an insertion of 12 bp (TTTTGACTTTTT) was found at the site of the excised *P*-element, presumably part of the *P*-element. No coding sequences of adjacent genes were affected by the deletion. Additionally, we tested the expression level of a neighboring sHsp gene, *Hsp22*, which can share regulatory sequences with *Hsp67Bc* and is crucial for the maintenance of mitochondria in *D. melanogaster* (Morrow et al., 2004). We found no difference between the obtained *Hsp67Bc* mutants and the control under normal conditions (see last paragraph of the Results section for information on *Hsp22* expression). The obtained flies carrying the deletion in the homozygous state were viable, fertile and phenotypically similar to the control. Hereafter in this paper, this *D. melanogaster Hsp67Bc*-null line is referred to as Hsp67Bc-0, whereas the control line (obtained by precise *P*-element excision from the same parent line) is called Hsp67Bc-2.

The effect of cold stress on *Hsp67Bc*-null *D. melanogaster* survival at various developmental stages

According to FlyBase (http://flybase.org/), mRNA and protein expression levels of *Hsp67Bc* are highest in late third instar larvae, and pupae of *D. melanogaster*. Therefore, we decided to investigate how cold treatment would affect the survival of Hsp67Bc-0 flies at larva and pupa stages of development, in addition to adults.

Drosophila individuals at a stage of wandering third instar larva (LL3), white prepupa (WPP), 12 h pupa [P (12 h)], 48 h pupa [P (48 h)] or adult (4-day-old males and females) were placed in a 0°C environment for 2 h (acute stress), 4 h (moderate stress) or 12 h incubation (prolonged stress), after which they were transferred back to 24.5°C to recover and/or continue development. The survival rates of larvae and pupae were computed from the number of eclosed individuals, and the survival rates of adult flies were determined 48 h after the cold treatment as a percentage of individuals alive. The results are presented in Fig. 3A. The response to the cold treatment depended on the stage in both

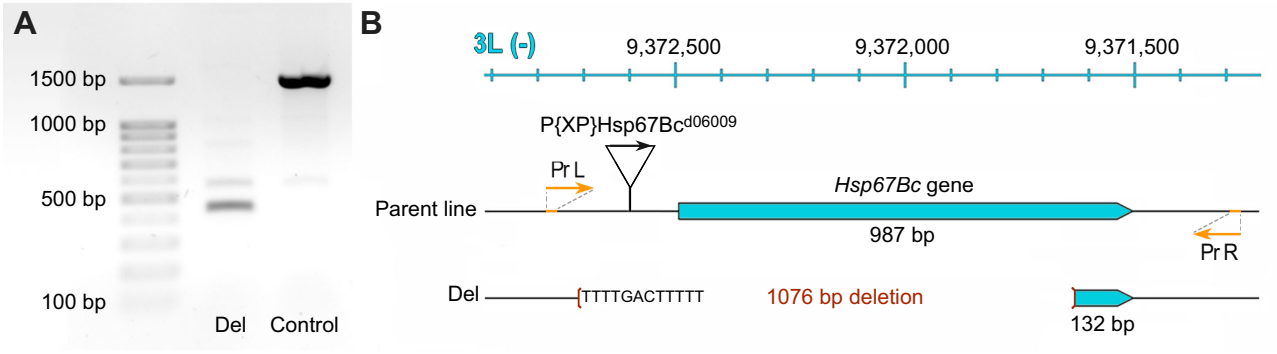

Fig. 2. PCR confirmation of the deletion in the *Hsp67Bc* gene, and an outline of this gene in control *Drosophila* and in the obtained mutant fly. (A) *Hsp67Bc* amplicon of the newly obtained mutant line (second lane) and control line (third lane). The amplicon, being 1508 bp in the control w^{1118} sample (Control), is ∼450 bp in the mutant sample (Del). (B) Illustration of the *Hsp67Bc* gene and the location of the *P*-element {XP} 115 bp upstream of this gene in parent line d06009 on chromosome 3L, as well as the size and location of the deleted region (indicated by dark red parentheses) in the resultant *Hsp67Bc*-null line (Del). A 12 bp insertion was left at the site of the excised *P*-element, presumably being its part. The positions of primers (Pr, orange arrows) used in the PCR are shown by orange segments on DNA strands.

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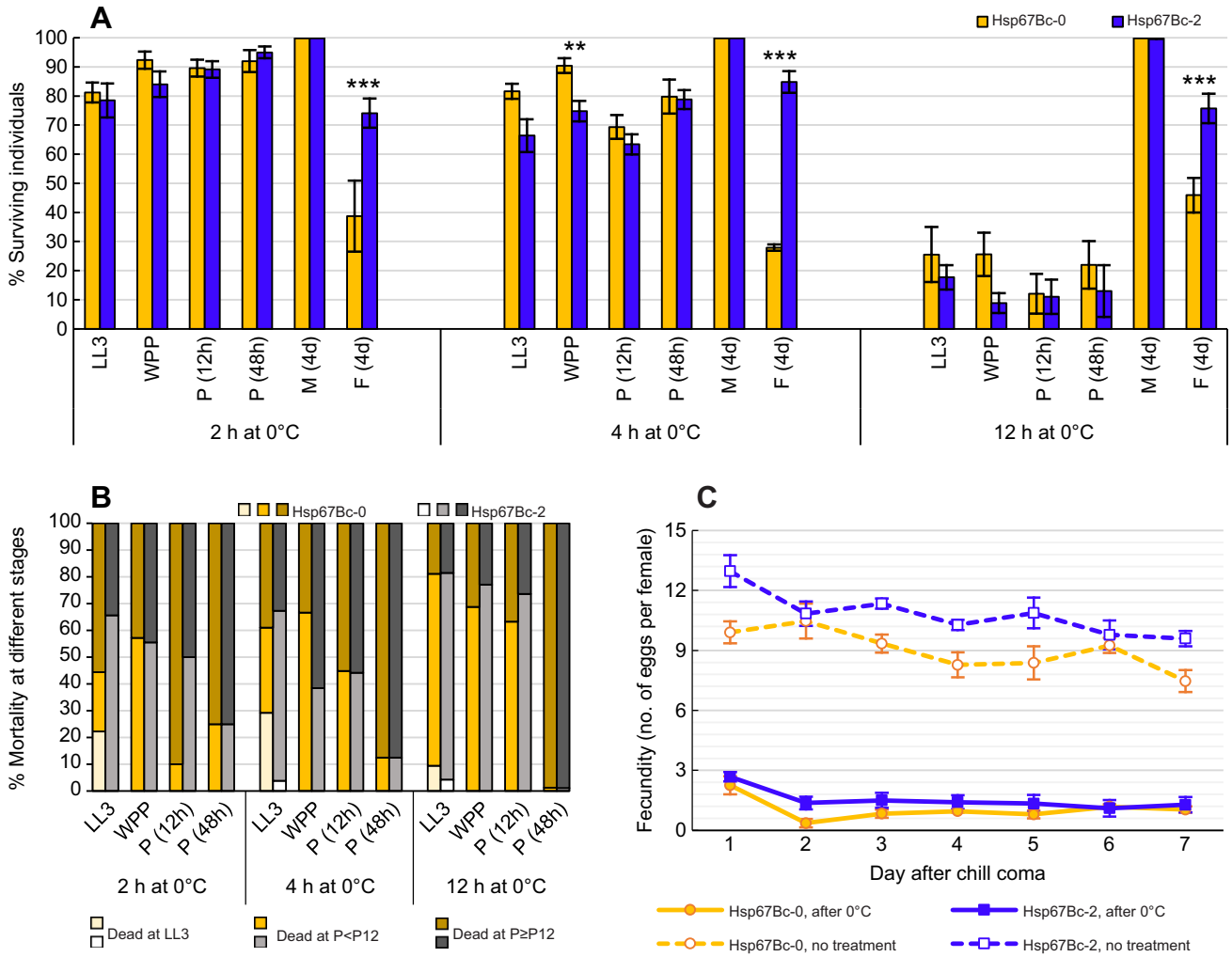

Fig. 3. Survival rates and fecundity of *Hsp67Bc*-null and control *Drosophila* after cold treatment of varying duration. Hsp67Bc-0: *Hsp67Bc*-null mutants; Hsp67Bc-2: control line; LL3: wandering L3 larvae; WPP: white prepupae; P (12h): 11–13 h pupae; P (48h): 47–49 h pupae; M (4d): 4-day-old adult males; F (4d): 4-day-old adult females. (A) The survival of Hsp67Bc-0 and Hsp67Bc-2 flies at different developmental stages after 2, 4 and 12 h of cold treatment (0°C). For LL3, the number of repeated experiments was 13 ($6\leq n\leq 14$) at 2 h cold treatment, 16 ($5\leq n\leq 26$) at 4 h, and six for Hsp67Bc-0 and seven for Hsp67Bc-2 ($8\leq n\leq 15$) at 12 h. For WPP, the number of repeated experiments was 12 for Hsp67Bc-0 and 13 for Hsp67Bc-2 ($3\leq n\leq 13$) at 2 h cold treatment, 13 ($4\leq n\leq 13$) at 4 h, and six for Hsp67Bc-0 and seven for Hsp67Bc-2 ($5\leq n\leq 12$) at 12 h. For P (12 h), the experiment was repeated five times at 2 and 12 h for Hsp67Bc-0 and 4 h cold treatment for Hsp67Bc-2, and six times for Hsp67Bc-2 at 2 and 12 h and Hsp67Bc-0 at 4 h, with N=20 in each. For P (48 h), the experiment was repeated five times at 2 h for Hsp67Bc-0 and 12 h for both lines, four times at 4 h for the Hsp67Bc-2 line, with N=20 in each; at 4 h cold treatment, the experiment was repeated eight times for the Hsp67Bc-0 line and nine times for Hsp67Bc-2, with N=20 in five of them and N=30 in the rest. In case of adult flies, the number of repeated experiments and individuals involved was the same as in recovery experiments plus data taken from fecundity experiments. (B) Percentage of flies that died at different stages of development (all larvae and pupae) after the end of 2, 4 and 12 h cold exposure. Left-hand bars, coloured in shades of gold, denote Hsp67Bc-0 mortality stages; right-hand grey bars denote Hsp67Bc-2 mortality stages. White and light gold segments indicate the proportion of individuals that died at the LL3 stage; grey and gold segments indicate the proportion of individuals that died as pupae before the P12 stage; dark grey and dark gold account for the proportion of individuals that died as pupae at P12 and later stages. (C) Fecundity of females after chill coma (0°C, 12 h; continuous lines) and under normal conditions (24.5°C, no treatment; dashed lines), measured each day for 1 week after the end of cold stress as the average number of eggs laid by each female in each vial. The initial number of vials was four for untreated flies (regarded as the control experiment repeated four times) and 10 for cold-treated flies (regarded as the experiment repeated 10 times) with N=8 and N=10 females in each vial, respectively. In A and C, data are means±s.e.m. Asterisks indicate significant difference between mutant and control lines (chi-squared test for survival and two-tailed heteroscedastic t-test for fecundity experiments: $^{**}0.001<P\leq 0.01$, $^{***}P\leq 0.001$).

control and *Hsp67Bc*-null flies. The survival rates declined with treatment time in larvae and pupae, whereas adult flies showed similar survival rates regardless of the duration of cold stress. Within the adult stage, strong sexual dimorphism was noted in terms of the survival after chill coma. No less than 98% of males of both lines were alive 48 h after each treatment, whereas the survival rates were lower and 1.6- to 3.0-fold different between the control and mutant females. The survival rates varied between 74.1±5.0% (2 h at 0°C) and 84.8±3.8% (4 h at 0°C) in Hsp67Bc-2 females, whereas only 27.9±1.1% (4 h at 0°C) to 45.9±5.9% (12 h at 0°C) of *Hsp67Bc*-null females stayed alive ($\chi^2\geq 49.80$, d.f.=1, $P<0.001$ in all the cold treatment groups). No deaths were observed after 48 h of recovery in adults.

After the acute and moderate cold stress, the survival rates of larvae and pupae were similar to those in adult males and control females, varying between 63.4±3.5% in Hsp67Bc-2 P (12 h) (moderate stress) and 95.0±2.0% in Hsp67Bc-2 P (48 h) (acute stress). Unlike adults, *Hsp67Bc*-null LL3 and pupae showed a

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tendency for an improved survival to adulthood (significant only at WPP after moderate cold exposure, χ^2=7.00, d.f.=1, P=0.008). After 12 h at 0°C, the survival of LL3 and pupae decreased dramatically, compared with that after acute and moderate cold stress. It did not exceed 25.5±9.5% in the Hsp67Bc-0 larvae and 17.7±4.2% in the Hsp67Bc-2 line, and among the tested pupal stages, the highest survival rate was observed in Hsp67Bc-0 WPP and Hsp67Bc-2 P (48 h): 25.5±7.4 and 13.0±8.9%, respectively. No significant difference was found between control and mutant *Drosophila* at LL3 and pupae stages after the prolonged cold stress.

The death of larvae and pupae did not necessarily occur exactly during or shortly after the cold treatment. Fig. 3B shows approximate stages [LL3, pupal stage <P12 (Bainbridge and Bownes, 1981) or ≥P12] at which all the individuals died after the exposure to 0°C. The mortality was delayed to the extent that, depending on the stage at which the cold exposure occurred, 20% (Hsp67Bc-0 LL3 after 12 h at 0°C) to almost 100% [P (48 h) after 12 h at 0°C in both lines] of the deceased individuals reached the ≥P12 stage, at which the imago is almost fully formed. Mutant and control LL3 individuals had significantly different survival to the pupal stage after short and moderate exposure to cold. Almost all the LL3 larvae survived to the pupal stage in the control line regardless of cold stress duration. Hsp67Bc-0 LL3 were significantly more cold susceptible: up to 29% (4 h at 0°C) of all the deceased individuals failed to proceed to the pupa stage. The overall survival from LL3 to pupa differed between the control and mutant lines, being 100% in the Hsp67Bc-2 line and 96.4±2.1% in mutants after short cold exposure (χ^2=5.95, d.f.=1, P=0.015), and 98.4±1.1% in Hsp67Bc-2 and 95.9±1.3% in Hsp67Bc-0 larvae after moderate cold stress (χ^2=6.39, d.f.=1, P=0.011).

Chill coma recovery of adult *Hsp67Bc*-null *D. melanogaster*

To test short-term recovery from cold stress, chill coma (0°C; 2, 4 or 12 h) experiments were conducted on 4-day-old Hsp67Bc-0 and Hsp67Bc-2 *D. melanogaster* individuals. Hsp67Bc-0 flies showed a slower recovery from the chill coma than the control (Fig. 4). Recovery dynamics of the mutant and control females differed significantly in all or in the majority of the experiment replicates ($1.34 \leq \lambda \leq 3.43$ in 2 h chill coma experiments, $0.001 < P < 0.06$; $\lambda \geq 1.94$, $P \leq 0.001$ in 4 h chill coma experiments; $\lambda \geq 1.80$, $P \leq 0.003$ in 12 h chill coma experiments). *Hsp67Bc*-null males recovered as fast as the control flies after 2 h of chill coma. Nevertheless, they showed slower recovery after two other cold treatment durations ($\lambda \geq 1.42$, $P \leq 0.036$). On average, all Hsp67Bc-2 males and females recovered from the comatose state within 40 min after the end of 2 and 4 h cold treatment and within 80 min after the end of 12 h cold stress, with females recovering slightly more slowly. Hsp67Bc-0 flies manifested similar patterns of recovery but with a 5–15 min delay.

The effect of cold stress on *D. melanogaster* fecundity

Under normal conditions (24.5°C, no treatment), *Hsp67Bc*-null female *Drosophila* showed lower fecundity than control flies (9.0±0.3 eggs per female compared with 10.8±0.3, averaged from the mean number of eggs laid by females during a 7-day period of registration, P<0.001), constituting 83.4% of that in the control line. After 12 h chill coma at 0°C, the fecundity of both Hsp67Bc-0 and Hsp67Bc-2 flies declined dramatically (Fig. 3C). Thus, the mean number of eggs laid by Hsp67Bc-2 females during the 7-day period after the end of the cold stress diminished approximately 7-fold to 1.5±0.1. In Hsp67Bc-0 females, this parameter went down more than 8-fold to as few as 1.1±0.2 eggs per female, constituting 69.5% of that in the control. Therefore, cold stress had similar negative effects on the fecundity of both control and mutant females.

The expression levels of several HSP genes, *Fst* and *stv* during cold exposure and recovery in *Drosophila* larvae, pupae and adults

Given that *D. melanogaster* survival and recovery were differently affected by the absence of the *Hsp67Bc* gene product, depending on the stage of development at which individuals were exposed to cold, we decided to measure expression levels of *Hsp67Bc* and some other HSP genes as well as *starvin* (*stv*) and *Frost* (*Fst*) in a search for possible candidates for Hsp67Bc function compensation. The Stv protein interacts with Hsp67Bc and is up-regulated in response to cold (Carra et al., 2010; Colinet and Hoffmann, 2010); *Fst* was also shown to be up-regulated after cold treatment (Colinet et al., 2010b). Among HSP genes, we selected *Hsp70* because it is a prominent heat- and cold-induced HSP in *Drosophila* (Goto and Kimura, 1998; Parsell et al., 1993), and four sHsps: *Hsp22*, *Hsp23*, *lethal (2) essential for life* [*l(2)efl*] and *Hsp67Ba*. *Hsp22* and *Hsp23* were chosen because they were proved to be involved in cold tolerance (Colinet et al., 2010c); *l(2)efl* is cold inducible and is abundantly expressed in the muscles of *Drosophila* larvae and adults (http://flybase.org/); and *Hsp67Ba* is prominently expressed in the central nervous system (CNS) of fruit fly larvae and pupae and larval carcass (http://flybase.org/). Gene expression was measured under normal conditions (24.5°C, no treatment), immediately after the end of cold treatment (0 min of recovery), and 90 min after the end of cold exposure in *D. melanogaster* at developmental stages that had a significant difference in chill coma survival, namely LL3, WPP and adult (4-day-old male and female) stages. The duration of cold treatment was 12 h for tested adults and 4 h for LL3 and WPP as 12 h chill coma had the same effect on the survival of adults as moderate and acute cold stress, but provided an opportunity to measure changes in gene expression levels shortly after the recovery from the comatose state. The prolonged cold stress was assumed to be too severe for larvae and pupae (less than a quarter of individuals of both genotypes survived to imago); therefore, 4 h cold stress was applied to LL3 and WPP instead.

Under normal conditions, the expression levels of the majority of assayed genes were the same between the control and *Hsp67Bc*-null flies at all tested stages of development, the exceptions being *stv* (which was 1.33-fold up-regulated in mutant LL3, P=0.020), *Hsp70* (which was 1.7-fold down-regulated in Hsp67Bc-0 adult females, P<0.001) and *Hsp23* (which was 1.6-fold down-regulated in Hsp67Bc-0 adult males, P=0.017) (Table 2). Notable, but not significantly different, tendencies towards up- or down-regulation were also observed for *Hsp70* and some other genes. The relative expression levels of the *Hsp67Bc* gene in the Hsp67Bc-2 line differed noticeably between the stages of development, being highest in WPP (2.8-fold higher than in LL3) and going down drastically by the adult stage (60-fold in males and 150-fold in females), in line with FlyBase data (http://flybase.org/). It is worth noting that *Hsp67Bc* expression significantly differed between males and females: its RNA levels were 2.5-fold lower in females (P=0.005). Sex-specific differences in expression were also present in some other assayed genes. For example, in control and mutant flies, the RNA levels of *stv*, *l(2)efl*, *Hsp67Ba* and *Fst* were higher or tended to be higher in males than in females. In females, only *Hsp23* was significantly up-regulated compared with males (7.9-fold higher in the control, and 16.4-fold higher in the *Hsp67Bc*-null line, P<0.001). These tendencies generally

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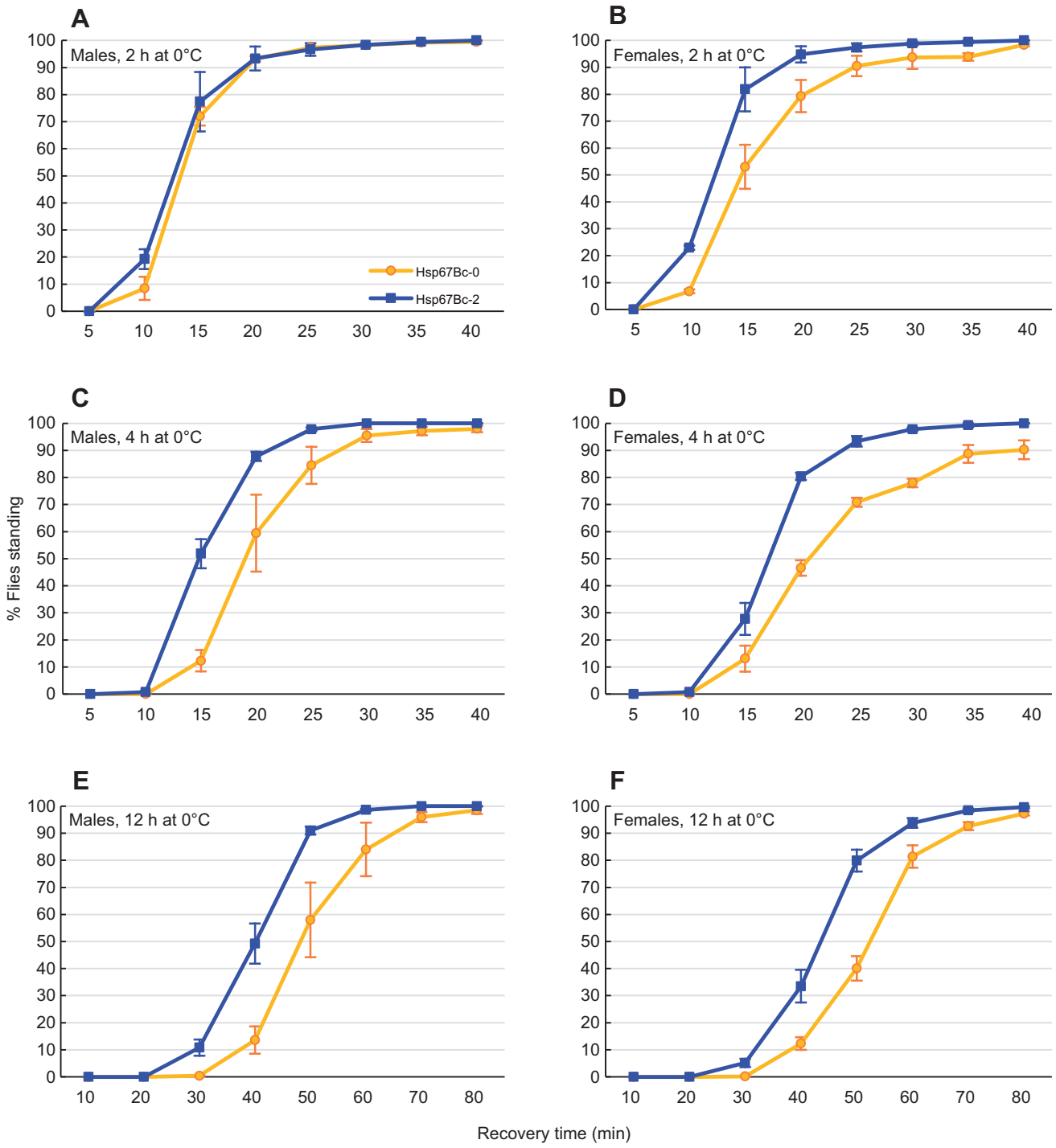

Fig. 4. The recovery curves of *Hsp67Bc*-null and control *Drosophila* males and females after 2, 4 and 12 h chill coma (0°C). Hsp67Bc-0: *Hsp67Bc*-null mutants; Hsp67Bc-2: control line. (A) Recovery curves of 4-day-old males after 2 h at 0°C. The recovery dynamics are not significantly different (Kolmogorov–Smirnov test here and in results presented in panels B–F; $\lambda \leq 1.17$). The experiment was conducted three times with N=90 Hsp67Bc-2 flies in each and N_1=60, N_2=87 and N_3=83 Hsp67Bc-0 flies. (B) Recovery curves of 4-day-old females after 2 h at 0°C. Hsp67Bc-0 females recover more slowly than Hsp67Bc-2 (λ_1=1.34, λ_2=1.42, λ_3=3.43, $0.001<P<0.06$; three experiments with N=90 Hsp67Bc-2 flies in each and N_1=75, N_2=N_3=90 Hsp67Bc-0 flies). (C) Recovery curves of 4-day-old males after 4 h at 0°C. Hsp67Bc-0 males recover more slowly than Hsp67Bc-2 males ($\lambda \geq 1.78$, $P \leq 0.004$; the experiment was repeated three times with N=90 Hsp67Bc-2 flies in each and N_1=73, N_2=75 and N_3=90 Hsp67Bc-0 flies). (D) Recovery curves of 4-day-old females after 4 h at 0°C. Hsp67Bc-0 females recover more slowly than Hsp67Bc-2 ($\lambda \geq 1.94$, $P \leq 0.001$; the experiment was repeated three times with N=90 Hsp67Bc-2 flies in each and N_1=84, N_2=N_3=90 Hsp67Bc-0 flies). (E) Recovery curves of 4-day-old males after 12 h at 0°C. Hsp67Bc-0 males recover more slowly than Hsp67Bc-2 ($\lambda_{1-5} \geq 1.42$, $P \leq 0.036$; λ_6=1.04, λ_7=0.83; the experiment was repeated seven times with N_{1-6}=90 and N_7=107 Hsp67Bc-2 flies and $N_{1-4,6}$=90, N_5=107 and N_7=62 Hsp67Bc-0 flies). (F) Recovery curves of 4-day-old females after 12 h at 0°C. Hsp67Bc-0 females recover more slowly than Hsp67Bc-2 females ($\lambda \geq 1.80$, $P \leq 0.003$; the experiment was repeated seven times with N_{1-6}=90 and N_7=107 Hsp67Bc-2 flies and N_{1-5}=90, N_6=98 and N_7=87 Hsp67Bc-0 flies). The registration time intervals were 5 min for 2 and 4 h chill coma experiments and 10 min for 12 h chill coma experiments. Data are means±s.e.m.

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Table 2. Expression levels of the assayed genes in the mutant (Hsp67Bc-0) and control (Hsp67Bc-2) flies at different stages of development under normal conditions (24.5°C, no treatment), with normalization to *αTub84B* levels

	Wandering L3			White prepupae			Adult males			Adult females		
	Hsp67Bc-2	Hsp67Bc-0	0/2	Hsp67Bc-2	Hsp67Bc-0	0/2	Hsp67Bc-2	Hsp67Bc-0	0/2	Hsp67Bc-2	Hsp67Bc-0	0/2
Hsp67Bc	7.57±2.70	–	–	21.04±3.35	–	–	0.35±0.02	–	–	0.14±0.01	–	–
Stv	3.67±0.14	4.87±0.46	1.33*	7.13±2.76	8.42±2.93	1.18	14.98±1.18	16.13±1.05	1.08	6.68±0.87	6.81±0.50	1.02
Hsp67Ba	4.09±0.52	3.34±0.51	0.82	10.32±1.09	8.88±0.59	0.86	0.56±0.02	0.62±0.05	1.11	0.25±0.02	0.22±0.01	0.88
Hsp70	3.99±2.15	3.00±1.17	0.75	22.67±2.13	27.91±2.49	1.23	6.55±2.12	3.57±0.58	0.55	4.75±0.47	2.75±0.28	0.58‡
Hsp22	1.88±0.66	1.66±0.43	0.89	1.95±0.22	1.45±0.25	0.74	6.43±0.25	6.87±1.79	1.07	7.38±0.54	6.76±0.50	0.92
Hsp23	31.19±14.97	33.42±27.63	1.07	222.58±65.64	168.52±27.24	0.76	5.65±0.14	3.44±0.08	0.61*	46.02±7.61	57.35±7.44	1.25
l(2)efl	92.54±35.85	99.64±27.24	1.08	8.69±2.34	12.70±3.19	1.46	18.64±0.92	22.52±1.31	1.21	8.66±0.11	9.73±0.57	1.12
Fst	4.55±1.13	4.69±1.05	1.03	0.71±0.17	0.81±0.06	1.13	0.18±0.06	0.29±0.03	1.62	0.11±0.00	0.11±0.01	0.99

The results in all but '0/2' are (means±s.e.m.)×10^2; in '0/2' columns, mutant-to-control expression level ratios are presented. *Difference at $0.01<P\leq0.05$; ‡$P\leq0.001$.

persisted after the cold stress (Fig. 5). In addition to the mentioned genes, the *Hsp70* level was 10-fold higher in control males than females ($P<0.001$) and showed a similar tendency in the mutant line (2.7-fold, $P=0.062$) immediately after the cold stress. Taken together, these sex-specific differences in the expression levels of the stress response genes after the exposure of flies to cold may account for the slight delay in recovery from chill coma in adult females compared with males.

The expression of the majority of investigated genes increased only at 90 min after the end of cold treatment (Fig. 5; Table 3). Expression levels of *stv* and *Hsp70* in the mutant females were 2.4- and 3.1-fold higher than those in the control females at 0 min of recovery, respectively ($P<0.001$). However, the *stv* level in the control females increased faster by 90 min of recovery (1.7-fold higher in Hsp67Bc-2 females compared with the mutants at 40 min of recovery, $P<0.001$, data not shown).

Ninety minutes after the end of cold treatment, the highest expression levels of all the assayed genes were observed (Fig. 5; Table 3). The most up-regulated genes were *Hsp70* and *Fst* in adults and *Hsp22* in LL3 larvae. The greatest increase of expression was observed for *Hsp70* (64-fold in the control males, $P=0.065$; 170-fold in Hsp67Bc-0 males, $P<0.001$; 99-fold in the control females, $P=0.032$; and 148-fold in the mutant females, $P<0.001$). It is worth noting that the majority of genes were differently affected by cold stress, depending on the developmental stage during the treatment. *Hsp67Bc* expression was higher in LL3 larvae and WPP than in adults and did not significantly change in response to cold stress, but increased 2.5-fold in males ($P=0.037$) and 6.1-fold in females ($P=0.011$). Although the expression level of *Hsp67Bc* was similar between males and females at 90 min of recovery, the up-regulation was delayed in females (Fig. 6). Thus, in females, *Hsp67Bc* RNA levels increased exponentially throughout the 25, 40 and 90 min recovery registration points, whereas in males, a close to a maximum level of RNA was reached by 25 min of recovery. The expression level of *Hsp67Bc* decreased between 90 min and 3 h after cold treatment (Fig. 6).

A few significant differences in RNA levels between the two *Drosophila* lines at 90 min of recovery were discovered for genes *stv*, *Hsp70*, *Hsp22* and *Fst* at different developmental stages, all of which were up-regulated in the mutant line compared with the control line (Fig. 5). The *Hsp70* RNA level was 1.7-fold higher in the adult males ($P<0.001$), the *Fst* level was elevated 1.2-fold in adult females ($P<0.001$), *stv* was up-regulated 1.3-fold in adult males ($P<0.001$) and 1.7-fold in WPP ($P=0.024$), and Hsp22 was 1.3-fold higher in WPP ($P=0.043$). It can be assumed that these increases in the expression of several cold stress response genes may be involved in compensation of the functions of the absent *Hsp67Bc* gene product.

DISCUSSION

Cold stress tolerance in *D. melanogaster* at different stages of development

In this study, we found that *Hsp67Bc*-null *Drosophila* larvae, pupae and adults have varying cold stress tolerance. Under acute (2 h at 0°C), moderate (4 h at 0°C) and chronic (12 h at 0°C) stress, the deletion in *Hsp67Bc* gene reduced cold tolerance in adults and wandering L3 larvae but not in white prepupae and pupae. Each stage of development showed distinct sensitivity to cold exposure. For instance, adult *Hsp67Bc*-null flies recovered from chill coma more slowly than the individuals with intact gene copies (Fig. 4), and females showed the most pronounced reduction in survival (1.6- to 3.0-fold lower than in the control line; Fig. 3A). *Hsp67Bc*-null LL3 larvae also featured a reduced survival to pupal stage after cold stress compared with the control individuals; however, the difference was not that prominent. *Hsp67Bc* deletion did not affect the survival rate after cold stress in late pupae and even slightly increased the survival in early pupae (Fig. 3A). We assume that this phenomenon is linked with the physiology of the corresponding stages. Larvae are characterized by extensive feeding, which is necessary for the accumulation of nutrients and energy to be used at the subsequent pupal stage; therefore, the metabolism slowdown caused by chill coma must have its consequences later on. Accordingly, we observed a slight decrease in survival to pupa at the late L3 stage and a delayed mortality at the pupal stage up to >P12 (Fig. 3B). Pupae are unable to feed, and the energy and supplies for the metamorphosis are provided via autophagic cell death, which removes most of the larval cells (Aguila et al., 2007). According to Merkey et al. (2011), metabolic rates decrease rapidly in pupae during the first 24 h and remain low until shortly before eclosion. Together with these processes, detrimental effects of cold stress (metabolic slowdown as well as ion and osmotic balance disruption that may eventually lead to cell death) either have no notable impact on the metamorphosis or have a delayed effect. Our data are consistent with the findings of Merkey et al. (2011), who reported that at the pupal stage, metabolic rates are independent of temperature fluctuations during the first two-thirds of pupal development.

Aside from survival, the imago stage can be characterized by sexual dimorphism in chill coma recovery speed. For example, females recovered more slowly than males in both the mutant and control lines, even after the shortest cold treatment (Fig. 4).

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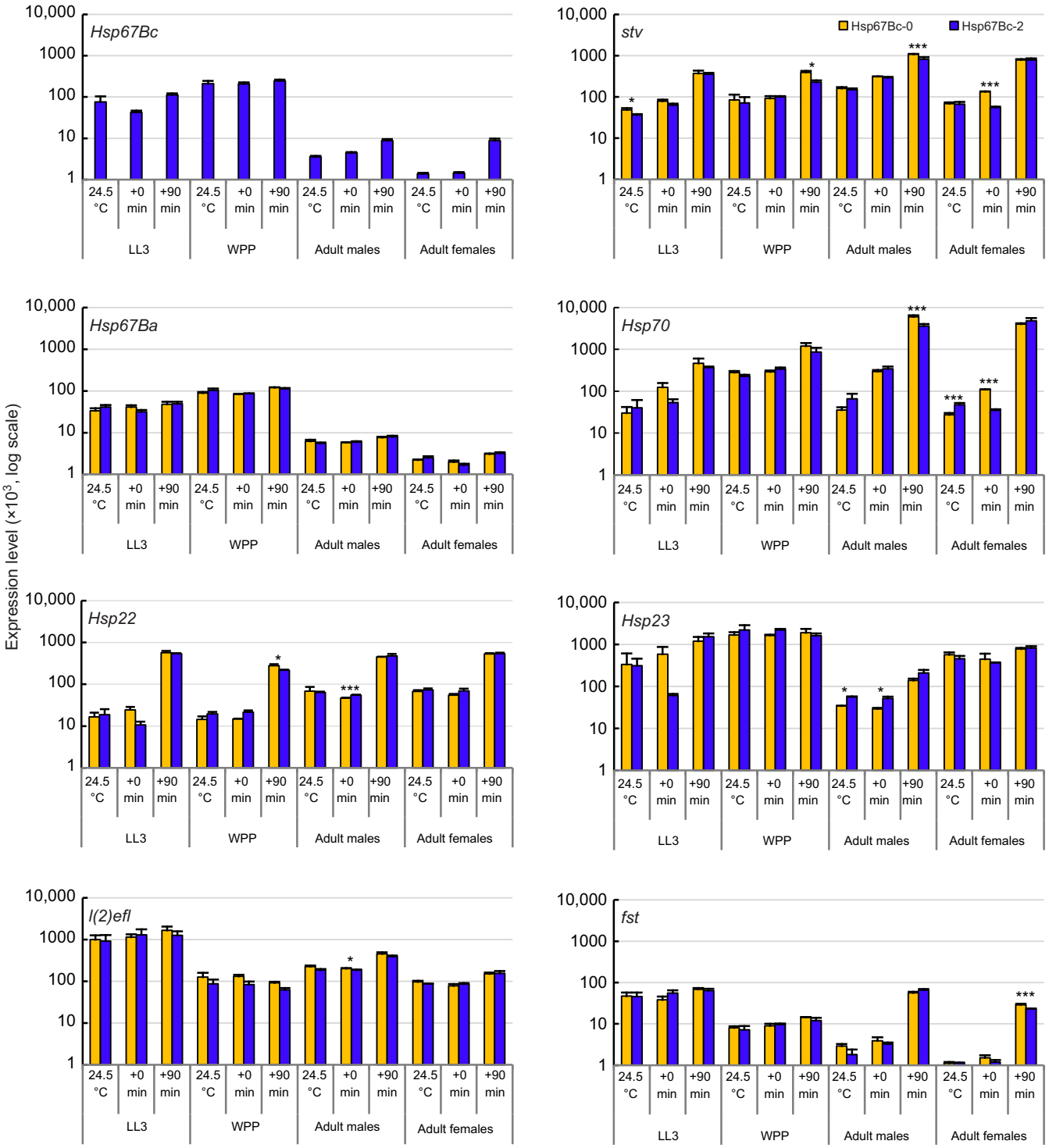

Fig. 5. Expression levels of *Hsp67Bc*, *stv*, *Fst* and chosen HSP genes under normal conditions and during recovery after the exposure to 0°C in Hsp67Bc-0 and Hsp67Bc-2 *Drosophila* individuals at different stages of development. Hsp67Bc-0: *Hsp67Bc*-null mutants; Hsp67Bc-2: control line; LL3: wandering L3 larvae; WPP: white prepupae; 'Adult males': 4-day-old adult males; 'Adult females': 4-day-old adult females; 24.5°C: normal conditions, no cold treatment; +0 min: 0 min of recovery after cold treatment (0°C); +90 min: 90 min of recovery after cold treatment (0°C). The duration of cold treatment was 4 h for larvae and prepupae and 12 h for adult flies. The gene expression levels are normalized to those of α*Tub84B* and shown as (means±s.e.m.)×10^3 on a logarithmic scale. For each data point, N=3. Asterisks indicate a significant difference in gene expression between mutant and control lines; *0.01<P≤0.05; ***P≤0.001.

We believe one of the causes to be the difference in the metabolome between males and females. A variety of studies indicate that the *Drosophila* metabolome is sex and age dependent, with sexual dimorphism increasing throughout development (Hoffman et al., 2014; Ingleby and Morrow, 2017). This notion is also in line with our findings about the difference in the expression of several genes between males and females (Table 2).

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Table 3. Changes in expression levels of the assayed genes throughout the recovery phase (0 and 90 min of recovery from 0°C) in *Hsp67Bc*-null (Hsp67Bc-0) and control (Hsp67Bc-2) *D. melanogaster* at different stages of development

	Wandering L3		White prepupae		Adult males		Adult females	
	+0 min	+90 min	+0 min	+90 min	+0 min	+90 min	+0 min	+90 min
Hsp67Bc-2								
Hsp67Bc	0.67	1.75	1.00	1.18	1.26§	2.45*	1.04	6.09*
Stv	1.68	9.29‡	1.60	3.63*	1.97§	5.49§	0.85	11.60*
Hsp67Ba	0.78	1.19	0.84	1.10	1.06	1.42*	0.68*	1.30
Hsp70	1.80	12.68‡	1.45	3.51	6.11§	63.90	0.74	98.85*
Hsp22	0.67	35.24§	1.12	11.65*	0.86*	7.41*	0.94	7.06§
Hsp23	0.35	8.41‡	1.08	0.78	0.91	3.56*	0.82*	1.81§
l(2)efl	1.50	1.51	1.00	0.76	1.00	2.07	0.99	1.77§
Fst	1.42	1.87	1.48	1.91	1.91*	36.40*	1.01	20.50§
Hsp67Bc-0								
Hsp67Bc	–	–	–	–	–	–	–	–
Stv	1.63§	7.49*	1.20	5.14	1.96§	6.79§	1.96*	11.37§
Hsp67Ba	1.25	1.42	0.93	1.36*	0.93	1.24	0.89	1.40§
Hsp70	4.28*	16.50*	1.04	4.13*	8.36§	170.09§	4.02§	147.74§
Hsp22	1.50	36.20§	1.04	20.17	0.74	7.16§	0.82	7.96*
Hsp23	2.48	7.65	0.99	1.14	0.84	3.96§	0.68	1.38*
l(2)efl	1.24	1.73	1.09	0.78	0.90	2.00§	0.79	1.54§
Fst	0.83	1.55	1.08	1.91	1.15	18.22§	1.31*	24.66*

The data are presented as means normalized to the expression levels under normal conditions (24.5°C, without cold exposure). *Difference at $0.01<P\leq0.05$; ‡$0.001<P\leq0.01$; §$P\leq0.001$.

Differences in gene expression

In addition to *Hsp67Bc*, we measured the expression of several genes, namely *Hsp70*, *Hsp22*, *Hsp23*, *Hsp67Ba*, *l(2)efl*, *stv* and *Fst*, in control and *Hsp67Bc*-null flies under normal (24.5°C) conditions as well as immediately after and 90 min after the end of 4 or 12 h cold treatment at 0°C (Tables 2 and 3; Fig. 5). As expected, we detected almost no statistically significant difference in the expression levels of the chosen genes between the mutant and control lines, with the exception of slight up-regulation of *stv* in mutant L3 larvae and down-regulation of *Hsp70* and *Hsp23* in adult females and adult males, respectively. The highest *Hsp67Bc* expression was observed at the pupal stage, whereas it was more than 60-fold lower in adults, in agreement with FlyBase data (http://flybase.org/). After the cold exposure, however, the expression of *Hsp67Bc* did not significantly change in pupae, tended to rise in the larvae, and dramatically increased in adults in a sex-specific manner. For instance, in males, *Hsp67Bc* expression was slightly elevated immediately after 12 h of the cold stress and reached a 2.5-fold increase by 90 min of recovery (Fig. 6). In females, the gene expression materialized with a lag but increased 6-fold by 90 min. This sexual dimorphism correlates with the different susceptibility of males and females to chill coma (Fig. 4). Apparently, the *Hsp67Bc* gene is more important for the recovery from chill coma in females, and its absence in mutants slows down the repair processes and reduces survival. The rate of recovery from chill coma depends on two processes: the recovery of CNS function and muscle polarization (Andersen and Overgaard, 2019). Although *Hsp67Bc* is primarily expressed in the CNS and Malpighian tubules (http://flybase.org/), the Hsp67Bc protein is also found in muscles (Carra et al., 2010). The deletion in this gene can impair both points of

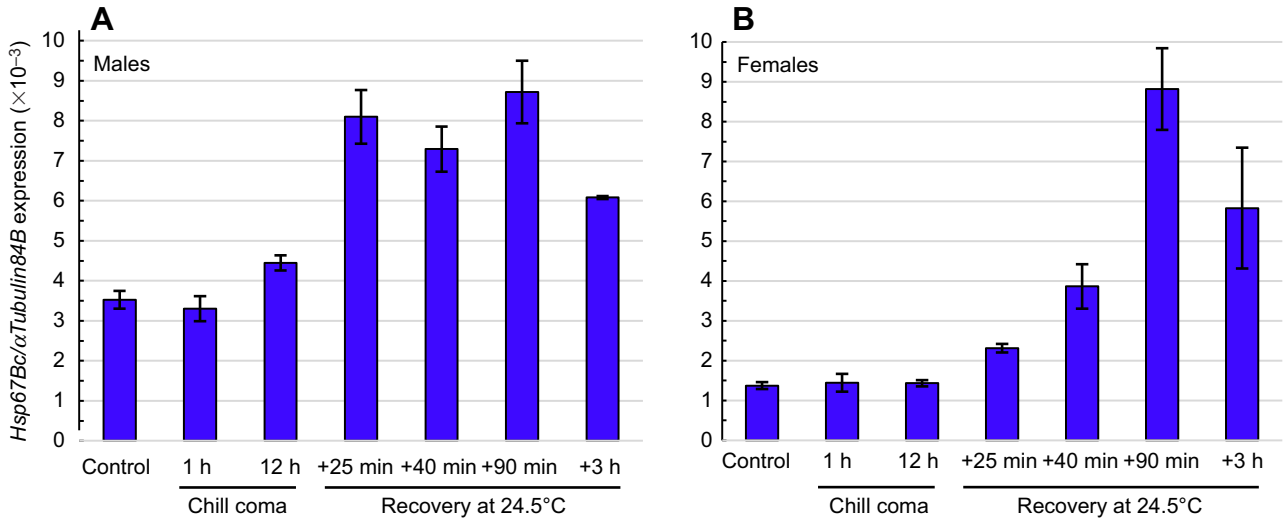

Fig. 6. The detailed expression pattern of *Hsp67Bc* during 12 h chill coma and the recovery phase in *Drosophila*. Control: 24.5°C, no cold treatment; 1 h: 1 h at 0°C; 12 h: 12 h at 0°C, corresponding to +0 min in Fig. 5; +25 min: 25 min of recovery; +40 min: 40 min of recovery; +90 min: 90 min of recovery; +3 h: 3 h of recovery. (A) *Hsp67Bc*/*αTub84B* expression ratio at different time points during cold stress and recovery in 4-day-old control males (no *Hsp67Bc* expression was detected in *Hsp67Bc*-null flies). (B) Same as in A, in 4-day-old control females. For each data point, N=3. Data are means±s.e.m.

neuromuscular recovery. Genes *Hsp23* and *l(2)efl*, which are abundantly expressed in the CNS and muscles, respectively, turned out to be up-regulated during the recovery phase (90 min after cold treatment in our study). By contrast, no differences in the expression of these genes were found between the control and *Hsp67Bc*-null lines (Fig. 5), implying their Hsp67Bc-independent functioning in chill coma recovery. Additionally, we assessed RNA levels of another sHsp gene, *Hsp67Ba*, which partially shares the expression pattern with *Hsp67Bc*, and found no significant correlation of *Hsp67Ba* levels with cold treatment, recovery phase, developmental stage and sex. Therefore, *Hsp67Ba* is unlikely to be a cold-tolerance gene.

Furthermore, we measured the expression of genes that have been shown to be involved in the cold stress response (Colinet and Hoffmann, 2010; Colinet et al., 2010b,c): *Hsp22*, *Hsp23*, *Hsp70*, *stv* and *Fst* (Tables 2 and 3; Fig. 5). As expected, all these genes proved to be up-regulated after cold exposure at all the fly developmental stages, although to varying degrees. The *stv* level was already increased during the cold exposure, reaching maximum expression at 90 min of recovery in both control flies and mutants, although with differing dynamics. Stv is a known participant of the stress response to cold exposure (Colinet and Hoffmann, 2010). These observations imply that Stv may be important for *Drosophila* protection from cold stress and for survival after chill coma. Genes *Hsp22*, *Hsp23* and *Fst* were found to be up-regulated only during the recovery period in both control and *Hsp67Bc*-null lines, thereby confirming their substantial role in the recovery of flies from a comatose state (Colinet et al., 2010b,c). The total *Hsp70* RNA level increased to a greater extent in *Hsp67Bc*-null mutants than control flies (170-fold higher in the mutant males versus 64-fold in the control males). Moreover, the up-regulation of the *Hsp70* gene was already noticeable during the chill coma, whereas only tendencies towards an increase in expression were seen in the control (Table 3; Fig. 5). The drastic up-regulation of *Hsp70* is consistent with other studies (Königer and Grath, 2018; MacMillan et al., 2016), and its more pronounced up-regulation in *Hsp67Bc*-null flies may denote its compensatory role in the response to cold stress in the absence of Hsp67Bc. It is possible that this compensatory mechanism allows *Hsp67Bc*-null *Drosophila* to survive, in contrast to *Hsp22*- and *Hsp23*-deficient flies. Colinet et al. (2010c) demonstrated that 24% of *Hsp22* and *Hsp23* knockout flies fail to recover from chill coma. Taken together, these data indicate that *Hsp22* and *Hsp23* play a more important part in chill coma recovery in *Drosophila*.

The presence of sex-specific differences in the expression of the assayed genes in both control and *Hsp67Bc*-null mutant flies is worth noting (Table 2; Fig. 5). As an example, immediately after the end of cold stress (0 min of recovery) *Hsp67Bc*, *Hsp67Ba*, *Hsp70*, *stv* and *Fst* had higher expression levels in males than females. This phenomenon can account for the difference in cold tolerance between males and females, as well as the dissimilarity of the sets of genes selected by the up-regulation extent after cold exposure in the studies by MacMillan et al. (2016) (males), Qin et al. (2005) (males), Vesala et al. (2012) (females) and Zhang et al. (2011) (females).

It remains unclear which functions exactly Hsp67Bc performs at the pupal stage under normal conditions. According to FlyBase (http://flybase.org/), the peak of *Hsp67Bc* gene expression level is reached at the pupal stage (which was also true in our study in white prepupae, compared with other assessed stages of development). It may be assumed that during metamorphosis, the *Hsp67Bc* gene product takes part in chaperone-assisted autophagic degradation of proteins in complex with its co-chaperone Stv (Arndt et al., 2010) or, on the contrary, in protein folding alongside other chaperones including sHsps. Apparently, these Hsp67Bc functions overlap with the roles of other proteins because *Hsp67Bc*-null homozygous *Drosophila* is viable and fertile.

Is *Hsp67Bc* a cold tolerance or a cold acclimation gene?

Data on the involvement of *Hsp67Bc* in cold tolerance are contradictory. MacMillan et al. (2016) noticed a 12-fold increase in *Hsp67Bc* expression in *D. melanogaster* males after 5-day-long acclimation at +5°C and therefore classified it as a cold acclimation gene. Vesala et al. (2012) investigated the acclimation in females belonging to two species of *Drosophila*: cold-tolerant *D. montana* as well as *D. virilis* having a more southern distribution range. Those authors discovered that after 5 days at +5°C, *Hsp67Bc* was 5.9-fold up-regulated in *D. virilis* but not in cold-tolerant *D. montana* and therefore, similarly, categorized it as an acclimation gene. Nonetheless, a similar study on *D. montana* and *D. virilis* females (Parker et al., 2015) did not confirm the participation of *Hsp67Bc* in cold acclimation. Here, we demonstrate that the expression of *Hsp67Bc* increases after the cold stress and decreases after 90 min of recovery (Fig. 6), pointing to its involvement in short-term recovery from cold stress. Our data are in line with the findings of Zhang et al. (2011) who investigated gene expression in *D. melanogaster* females at 6 h of recovery from chill coma and detected no changes in the *Hsp67Bc* level. These findings allow us to regard *Hsp67Bc* as a cold tolerance gene. We also discovered that the deletion in this gene results in a statistically significant delay in the chill coma recovery. Moreover, survival was impaired in *Hsp67Bc*-null larvae and adults within 2 days after the chill coma; this pattern also characterizes a short-term stress response.

Cold acclimation lasts for days or weeks during which the concentration of sugars, polyols and amino acids increases, thereby enhancing cryoprotective capacity of cells and altering their energy exchange, proline and glutathione metabolism, lipid composition, cell membrane permeability, and ion balance, all of which contribute to the protection of the organism against cold injury (Colinet and Hoffmann, 2012; Koštál et al., 2016; MacMillan et al., 2016; Overgaard and MacMillan, 2017; Overgaard et al., 2008). The response to cold stress or chill coma includes repair of cold-induced damage, specifically DNA and protein stabilization and repair, the removal of damaged proteins, and shifts in certain parameters of metabolism involving heat shock proteins and other stress-related genes (Colinet and Hoffmann, 2012; Colinet et al., 2010a; Parker et al., 2015; Zhang et al., 2011). The increase in the mortality rate after chill coma in *Hsp67Bc*-null *D. melanogaster* occurred with a delay. That is, the individuals did not die during the cold exposure or immediately after it; rather they died within days after being transferred to normal conditions, indicating an impaired repair process. Therefore, considering all the findings, we regard *Hsp67Bc* as a cold stress tolerance gene rather than cold acclimation gene.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.M., E.K., S.F.; Methodology: D.M., S.F.; Validation: D.M.; Formal analysis: D.M.; Investigation: D.M.; Resources: E.K., S.F.; Data curation:

D.M.; Writing - original draft: D.M., E.K., S.F.; Writing - review & editing: D.M., E.K., S.F.; Visualization: D.M.; Supervision: S.F.; Project administration: E.K.; Funding acquisition: E.K., S.F.

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References

Aguila, J. R., Suszko, J., Gibbs, A. G. and Hoshizaki, D. K. (2007). The role of larval fat cells in adult *Drosophila melanogaster*. *J. Exp. Biol.* **210**, 956-963. doi:10.1242/jeb.001586

Andersen, M. K. and Overgaard, J. (2019). The central nervous system and muscular system play different roles for chill coma onset and recovery in insects. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **233**, 10-16. doi:10.1016/j.cbpa.2019.03.015

Arndt, V., Dick, N., Tawo, R., Dreiseidler, M., Wenzel, D., Hesse, M., Fürst, D. O., Saftig, P., Saint, R., Fleischmann, B. K. et al. (2010). Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr. Biol.* **20**, 143-148. doi:10.1016/j.cub.2009.11.022

Bainbridge, S. P. and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **66**, 57-80.

Carra, S., Boncoraglio, A., Kanon, B., Brunsting, J. F., Minoia, M., Rana, A., Vos, M. J., Seidel, K., Sibon, O. C. M. and Kampinga, H. H. (2010). Identification of the *Drosophila* ortholog of HSPB8: implication of HSPB8 loss of function in protein folding diseases. *J. Biol. Chem.* **285**, 37811-37822. doi:10.1074/jbc.M110.127498

Colinet, H. and Hoffmann, A. (2010). Gene and protein expression of *Drosophila* Starvin during cold stress and recovery from chill coma. *Insect Biochem. Mol. Biol.* **40**, 425-428. doi:10.1016/j.ibmb.2010.03.002

Colinet, H. and Hoffmann, A. A. (2012). Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*. *Funct. Ecol.* **26**, 84-93. doi:10.1111/j.1365-2435.2011.01898.x

Colinet, H., Lee, S. F. and Hoffmann, A. (2010a). Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. *FEBS J.* **277**, 174-185. doi:10.1111/j.1742-4658.2009.07470.x

Colinet, H., Lee, S. F. and Hoffmann, A. (2010b). Functional characterization of the Frost gene in *Drosophila melanogaster*: importance for recovery from chill coma. *PLoS ONE* **5**, e10925. doi:10.1371/journal.pone.0010925

Colinet, H., Lee, S. F. and Hoffmann, A. (2010c). Knocking down expression of Hsp22 and Hsp23 by RNA interference affects recovery from chill coma in *Drosophila melanogaster*. *J. Exp. Biol.* **213**, 4146-4150. doi:10.1242/jeb.051003

Coulson, M., Robert, S. and Saint, R. (2005). *Drosophila* starvin encodes a tissue-specific BAG-domain protein required for larval food uptake. *Genetics* **171**, 1799-1812. doi:10.1534/genetics.105.043265

David, J. R., Gibert, P., Pla, E., Petavy, G., Karan, D. and Moreteau, B. (1998). Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. melanogaster*. *J. Therm. Biol.* **23**, 291-299. doi:10.1016/S0306-4565(98)00020-5

Goto, S. G. and Kimura, M. T. (1998). Heat- and cold-shock responses and temperature adaptations in subtropical and temperate species of *Drosophila*. *J. Insect Physiol.* **44**, 1233-1239. doi:10.1016/S0022-1910(98)00101-2

Hoffman, J. M., Soltow, Q. A., Li, S., Sidik, A., Jones, D. P. and Promislow, D. E. L. (2014). Effects of age, sex, and genotype on high-sensitivity metabolomic profiles in the fruit fly, *Drosophila melanogaster*. *Aging Cell* **13**, 596-604. doi:10.1111/acel.12215

Ingleby, F. C. and Morrow, E. (2017). Sexual dimorphism in the Drosophila melanogaster (Diptera: Drosophilidae) metabolome increases throughout development. *Eur. J. Entomol.* **114**, 249-256. doi:10.14411/eje.2017.030

Königer, A. and Grath, S. (2018). Transcriptome analysis reveals candidate genes for cold tolerance in *Drosophila ananassae*. *Genes (Basel)* **9**, 624. doi:10.3390/genes9120624

Koštál, V., Korbelová, J., Štetina, T., Poupardin, R., Colinet, H., Zahradníková, H., Opekarová, I., Moos, M. and Šimek, P. (2016). Physiological basis for low-temperature survival and storage of quiescent larvae of the fruit fly *Drosophila melanogaster*. *Sci. Rep.* **6**, 1-11. doi:10.1038/s41598-016-0001-8

Leemans, R., Egger, B., Loop, T., Kammermeier, L., He, H., Hartmann, B., Certa, U., Hirth, F. and Reichert, H. (2000). Quantitative transcript imaging in normal and heat-shocked Drosophila embryos by using high-density oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* **97**, 12138-12143. doi:10.1073/pnas.210066997

Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* **55**, 1151-1191. doi:10.1146/annurev.bi.55.070186.005443

Macdonald, S. S., Rako, L., Batterham, P. and Hoffmann, A. A. (2004). Dissecting chill coma recovery as a measure of cold resistance: evidence for a biphasic response in *Drosophila melanogaster*. *J. Insect Physiol.* **50**, 695-700. doi:10.1016/j.jinsphys.2004.05.004

MacMillan, H. A., Knee, J. M., Dennis, A. B., Udaka, H., Marshall, K. E., Merritt, T. J. S. and Sinclair, B. J. (2016). Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Sci. Rep.* **6**, 1-14. doi:10.1038/srep28999

Merkey, A. B., Wong, C. K., Hoshizaki, D. K. and Gibbs, A. G. (2011). Energetics of metamorphosis in *Drosophila melanogaster*. *J. Insect Physiol.* **57**, 1437-1445. doi:10.1016/j.jinsphys.2011.07.013

Morrow, G. and Tanguay, R. M. (2015). *Drosophila* small heat shock proteins: an update on their features and functions. In *The Big Book on Small Heat Shock Proteins* (ed. R. M. Tanguay and L. E. Hightower), pp. 579-606. Switzerland: Springer International Publishing.

Morrow, G., Battistini, S., Zhang, P. and Tanguay, R. M. (2004). Decreased lifespan in the absence of expression of the mitochondrial small heat shock protein Hsp22 in *Drosophila*. *J. Biol. Chem.* **279**, 43382-43385. doi:10.1074/jbc.C400357200

Myachina, F., Bosshardt, F., Bischof, J., Kirschmann, M. and Lehner, C. F. (2017). *Drosophila* β-tubulin 97EF is upregulated at low temperature and stabilizes microtubules. *Development* **144**, 4573-4587. doi:10.1242/dev.156109

Newman, C. E., Toxopeus, J., Udaka, H., Ahn, S., Martynowicz, D. M., Graether, S. P., Sinclair, B. J. and Percival-Smith, A. (2017). CRISPR-induced null alleles show that Frost protects *Drosophila melanogaster* reproduction after cold exposure. *J. Exp. Biol.* **220**, 3344-3354. doi:10.1242/jeb.160176

Overgaard, J. and MacMillan, H. A. (2017). The integrative physiology of insect chill tolerance. *Annu. Rev. Physiol.* **79**, 187-208. doi:10.1146/annurev-physiol-022516-034142

Overgaard, J., Sørensen, J. G., Petersen, S. O., Loeschcke, V. and Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J. Insect Physiol.* **51**, 1173-1182. doi:10.1016/j.jinsphys.2005.06.007

Overgaard, J., Tomčala, A., Sørensen, J. G., Holmstrup, M., Krogh, P. H., Šimek, P. and Koštál, V. (2008). Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster*. *J. Insect Physiol.* **54**, 619-629. doi:10.1016/j.jinsphys.2007.12.011

Parker, D. J., Vesala, L., Ritchie, M. G., Laiho, A., Hoikkala, A. and Kankare, M. (2015). How consistent are the transcriptome changes associated with cold acclimation in two species of the *Drosophila virilis* group? *Heredity (Edinb)* **115**, 13-21. doi:10.1038/hdy.2015.6

Parsell, D. A., Taulien, J. and Lindquist, S. (1993). The role of heat-shock proteins in thermotolerance. In *Molecular Chaperones* (ed. R. J. Ellis and R. A. Laskey and G. H. Lorimer), pp. 23-30. Dordrecht: Springer.

Ponton, F., Chapuis, M. P., Pernice, M., Sword, G. A. and Simpson, S. J. (2011). Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *J. Insect Physiol.* **57**, 840-850. doi:10.1016/j.jinsphys.2011.03.014

Qin, W., Neal, S. J., Robertson, R. M., Westwood, J. T. and Walker, V. K. (2005). Cold hardening and transcriptional change in *Drosophila melanogaster*. *Insect Mol. Biol.* **14**, 607-613. doi:10.1111/j.1365-2583.2005.00589.x

Rajamohan, A. and Sinclair, B. J. (2008). Short-term hardening effects on survival of acute and chronic cold exposure by *Drosophila melanogaster* larvae. *J. Insect Physiol.* **54**, 708-718. doi:10.1016/j.jinsphys.2008.01.011

Sinclair, B. J. and Roberts, S. P. (2005). Acclimation, shock and hardening in the cold. *J. Therm. Biol.* **30**, 557-562. doi:10.1016/j.jtherbio.2005.07.002

Vesala, L., Salminen, T. S., Laiho, A., Hoikkala, A. and Kankare, M. (2012). Cold tolerance and cold-induced modulation of gene expression in two *Drosophila virilis* group species with different distributions. *Insect Mol. Biol.* **21**, 107-118. doi:10.1111/j.1365-2583.2011.01119.x

von Heckel, K., Stephan, W. and Hutter, S. (2016). Canalization of gene expression is a major signature of regulatory cold adaptation in temperate *Drosophila melanogaster*. *BMC Genomics* **17**, 1-14. doi:10.1186/s12864-016-2866-0

Vos, M. J., Carra, S., Kanon, B., Bosveld, F., Klauke, K., Sibon, O. C. M. and Kampinga, H. H. (2016). Specific protein homeostatic functions of small heat-shock proteins increase lifespan. *Aging Cell* **15**, 217-226. doi:10.1111/acel.12422

Zhang, J., Marshall, K. E., Westwood, J. T., Clark, M. S. and Sinclair, B. J. (2011). Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster*. *J. Exp. Biol.* **214**, 4021-4029. doi:10.1242/jeb.059535