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RESEARCH ARTICLE

Rapid decline of cold tolerance at young age is associated with expression of stress genes in *Drosophila melanogaster*

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SUMMARY

Many endogenous factors influence thermal tolerance of insects. Among these, age contributes an important source of variation. Heat tolerance is typically high in newly eclosed insects, before declining dramatically. It is not known whether this phenomenon relates to cold tolerance also. In addition, the underlying mechanisms of this variation are unresolved. In this study, we tested whether cold tolerance declines in *Drosophila melanogaster* females aged from 0 to 5 days. We also assessed whether expression (basal and induced) of eight stress genes (*hsp22*, *hsp23*, *hsp40*, *hsp68*, *hsp70Aa*, *hsp83*, *Starvin* and *Frost*) varied post-eclosion in correspondence with changes found in cold tolerance. We report that cold tolerance was very high at eclosion and then it rapidly declined in young flies. *hsp23* and *hsp68* showed a dramatic age-related variation of basal expression that was associated with cold tolerance proxies. Significant age-related plasticity of cold-induced expression was also found for *hsp22*, *hsp23*, *hsp68*, *hsp70Aa*, *Frost* and *Starvin.* Induced expression of *hsp22* and *hsp70Aa* was high in newly enclosed phenotypes before declining dramatically, whilst opposite age-related patterns were found for *hsp23*, *hsp68*, *Starvin* and *Frost*. This study shows a marked within-stage variation in cold tolerance. The involvement of the stress genes in setting basal thermal tolerance is discussed.

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INTRODUCTION

Thermal tolerance of ectotherms, and its variability, have long been a central theme in the field of ecology, physiology and evolutionary biology. A wealth of endogenous factors can potentially influence thermal tolerance of insects (Bowler and Terblanche, 2008), but the role of intrinsic biological variation has often been overlooked (Bowler and Terblanche, 2008; Spicer and Gaston, 1999). Age and/or ontogeny are important sources of variation of thermal traits, and these factors may confound studies of temperature responses if unaccounted for (Bowler and Terblanche, 2008).

In insects, the most documented variation in thermal tolerance associated with ontogeny is that attributed to variation among life stages. However, within-life-stage effects may also contribute to important variation (Bowler and Terblanche, 2008). The experimental gerontology literature typically predicts a reduction in general stress resistance with senescence (Grotewiel et al., 2005; Minois and Le Bourg, 1999). However, age-dependent effects unrelated to senescence have also been reported. For instance, heat tolerance is typically high at eclosion, then it decreases dramatically at young age to a stable lower level; therefore, it is not a senescence effect *per se* (Bowler, 1967; Bowler and Hollingsworth, 1965). There are a number of reports for rapid decline of heat tolerance in holometabolous insects (Bowler, 1967; Bowler and Hollingsworth, 1965; Davison, 1969; Krebs et al., 1998; Sørensen and Loeschcke, 2002; Pappas et al., 2007). The rate of this process is temperature-dependent (Davison, 1969), so that it may, in some cases, obscure the effect of thermal acclimation. This was the case in the blowfly, where an apparent paradoxical effect of acclimation was found, with young flies maintained at low temperatures being more heat-tolerant than counterparts maintained at high temperature (Davison, 1969). It is not known whether this biological variation is specific to heat tolerance or whether it also impacts on cold tolerance. Jensen et al. investigated cold tolerance across developmental stages in *Drosophila melanogaster* and provided early signs for higher cold tolerance of young females (Jensen et al., 2007). However, no study has precisely addressed whether variation at young age affects cold tolerance. It has been assumed that changes in physiological performance after transition to a new life stage could represent a 'carry-over' of the temperature tolerance from a previous life stage (Bowler and Hollingsworth, 1965; Bowler and Terblanche, 2008). More data are needed to decipher this phenomenon, and understanding whether cold tolerance is also affected at young age would represent a critical step.

The underlying mechanisms for high heat tolerance of newly enclosed insects are not yet resolved. Because heat shock proteins (Hsps) regulate stress tolerance and lifespan (Sørensen et al., 2003; Tower, 2009), these molecular chaperones are prime candidates for deciphering this phenomenon. A reduction in the expression of the protein Hsp70 was found to accompany the age-related decline in

heat tolerance in *D. melanogaster* (Sørensen and Loeschcke, 2002; Pappas et al., 2007). In insects, there has been a long-standing focus on the protein Hsp70, which remains the most commonly studied stress protein (Sørensen et al., 2003; Sørensen and Loeschcke, 2007). Even if expression level of Hsp70 is a good indicator of the whole inducible stress response, studied alone it might give an incomplete picture of the organism's stress response (Colinet et al., 2010a). Indeed, Hsp70 is known to interact with a network of other Hsps (Bettencourt et al., 2008; Tower, 2011). Therefore, if Hsp70 displays a mild modulation under a specific condition, it is possible that changes in the expression of other Hsp proteins might occur and might be overlooked (Sørensen and Loeschcke, 2007). Therefore, it is essential to analyse expression of other responsive Hsps (genes and/or proteins). Several *hsp* genes are expressed during the recovery from cold stress in *D. melanogaster* (Colinet et al., 2010a; Colinet and Hoffmann, 2012), and RNAi experiments have demonstrated that expression of some of these genes is essential for insect cold tolerance and recovery from cold stress (Colinet et al., 2010b; Kostál and Tollarova-Borovanska, 2009; Rinehart et al., 2007). Thus, cold-responsive *hsp* genes are good potential candidates for investigating the variation of molecular stress response at young age. Whether the levels of *hsp* genes expressed constitutively (i.e. basal level) or in response to stress exposure (i.e. induced level) influence, or even determine, thermal tolerance is the question that we undertake in this study.

Most studies on *Drosophila* use young adults (approximately one week old) as their experimental reference. It is therefore implicitly considered that during the first week of adult life all aspects of the organism's biology remain relatively stable, but this assumption is not warranted (Technau, 1984; Ford et al., 1989). The present work was designed to (1) investigate whether cold tolerance declines at young age and (2) assess whether expression levels (basal and induced) of various stress genes vary at young age. In addition to six *hsp* genes (i.e. *hsp22*, *hsp23*, *hsp40*, *hsp68*, *hsp70Aa* and *hsp83*) that are known to be cold-responsive (Colinet et al., 2010a; Colinet and Hoffmann, 2012), we also analysed expression patterns of two other genes involved in the cold stress response in *D. melanogaster*: *Starvin* (*Stv*) (Colinet and Hoffmann, 2010) and *Frost* (*Fst*) (Colinet et al., 2010c).

MATERIALS AND METHODS

Fly culture

We conducted our experiments on a mass-bred *Drosophila melanogaster* line derived from the mix of two wild populations collected in October 2010 at Plancoët and Rennes (Brittany, France). The flies were maintained in the laboratory in 200 ml bottles at 25±1°C (16 h:8 h L:D) on standard fly medium consisting of sugar, brewer's yeast and agar, as described previously (Colinet and Hoffmann, 2012).

Experimental design

To generate flies for the experiments, groups of 30 six-day-old females were allowed to lay eggs in 200 ml bottles containing standard food during a restricted period of 12 h. This semi-controlled procedure allowed the flies to develop under uncrowded conditions at 25±1°C (16 h:8 h L:D). Upon emergence, virgin flies of less than 6 h old were collected. They were sexed visually without CO_2 and only females were maintained in food vials at a density of 30 flies at 25±1°C (16 h:8 h L:D). Females were then transferred to fresh food every day. Virgin females aged 0 (less than 6 h), 1, 2, 3, 4 and 5 days old were tested for cold tolerance and gene expression. We restricted the analyses to the first five days of age because it was shown that most changes in Hsp expression occurred between days 0 and 3, with little additional changes after day 3 (Pappas et al., 2007).

Cold tolerance assessment

Different metrics were used to investigate cold tolerance. For each age group, recovery time following a nonlethal chronic cold stress was measured as previously described (Colinet et al., 2010a). Briefly, for each age, 50 females were exposed to 0°C for 14 h by placing a vial in a cold incubator (Model MIR-153; SANYO Electric Co. Ltd, Munich, Germany). Flies were then allowed to recover at 25±1°C (16 h:8 h L:D), and chill coma recovery times were individually recorded. Flies were considered recovered when they stood up. Data were used to generate temporal recovery curves, which were compared using Mantel–Cox analysis (Colinet et al., 2010a). Survival after chronic stress at 0°C for 14 h was also measured for each age group. After the stress exposure, five replicated pools of 20 females (i.e. a total of 100 flies) were returned to 25±1°C (16 h:8 h L:D) on food, and survival was scored after 24 h. Mean survival was compared among ages using χ^2 test. Age-related changes in critical thermal minimum (CT_{min}) were also investigated following the method described previously (Lalouette et al., 2010). An ethylene glycol jacketed glass cylinder (35×5 cm) was used. Temperature in the cylinder was controlled by circulating ethylene glycol from a programmable bath (Haake F3 Electron, Karlsruhe, Germany). Flies were cooled from 20°C to the CT_{min} at 0.75 deg min^{-1}. Upon entering chill coma, flies fell out, and the temperature inside the column was recorded using a thermocouple (accuracy of ±0.15°C). For each age group, 24 females were tested. Mean CT_{min} values were compared using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) comparison tests.

Molecular analyses

For stress genes expression, we measured basal (i.e. constitutive) and cold-induced expressions separately. Basal expression was measured in untreated females of each age group, while induced expression was measured during the recovery (at 25°C) following a chronic cold exposure (0°C for 14 h). The analysed genes show a peak of expression after 2 h of recovery following cold stress (Colinet et al., 2010a; Colinet et al., 2010c; Colinet and Hoffmann, 2010); therefore, cold-stressed flies were allowed to recover for 2 h before they were snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction. Three different biological replicates consisting of 25 females were used for gene expression analyses.

RNA isolation and cDNA synthesis

Total RNA extraction was performed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Samples were treated with DNase I (Ambion, Austin, TX, USA) following the manufacturer's instructions, and quantified by spectrophotometry at 260 nm (BioPhotometer, Eppendorf, Germany). Single-stranded cDNAs were synthesised from total RNA (5 µg) with Superscript II reverse transcriptase (Gibco BRL, Invitrogen) and a buffer containing dNTPs and $Oligo(dT)_{18}$ primer. The reaction mixture was heated (95°C, 5 min) before adding RNase OUT enzyme, and ultrapure water to a final volume of 20 µl. The reaction mixture was incubated for 50 min at 42°C, and then 10 min at 70°C.

Reference gene selection and primer design

Expression of five different reference genes (*Pgk*, *Rp49*, *RpL13*, *RpS20* and *Tbp*) was first tested. In order to determine the best

reference gene, the average cycle threshold value (Ct-value) derived from qPCRs (see below) of each triplicate reaction was used for analysis with BestKeeper program (Pfaffl et al., 2004). *RpL13* displayed the most consistent expression and was selected as the best reference gene among all experimental conditions. Expression of six cold-responsive *hsp* genes (*hsp22*, *hsp23*, *hsp40*, *hsp68*, *hsp70Aa* and *hsp83*) and two other cold-responsive genes (*Fst* and *Stv*) was tested. Specific qPCR primers (supplementary material Table S1) were designed using Eprimer3 software (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::eprimer3), and optimal primer annealing temperatures were optimized using qPCR tests.

qRT-PCRs

Real-time quantitative PCRs (qRT-PCRs) were performed on the LightCycler480 Detection System (Roche Applied Science, Meylan, France). Each reaction consisted of 6 µl Absolute Blue SYBR Green Fluor (Thermo Scientific, Waltham, MA, USA), 4 µl cDNA (25 ng µl^{-1}), 1 µl of each primer (10 µmol l^{-1}) and 1 µl of ultrapure water. The PCR program consisted of an initial denaturation (95°C, 5 min), then 40 cycles of 95°C for 10 s, 60°C for 15 s, 72°C for 15 s. Each run included a negative control (water) and a fivefold dilution series of pooled cDNA (from all conditions), which produced standard curves that confirmed high PCR efficiencies (90–100%). Each reaction was run in triplicate (technical replicate) for each of the three independent biological replicates. Expression levels were analysed with LightCycler480 software (Roche), and the Ct-values were determined for the reference and candidate genes. The average Ct-value of each triplicate reaction was used to normalise the candidate gene expression level to the geometric mean of the reference gene's level in Q-Gene (Simon, 2003). Basal normalised expression was expressed relative to expression of day 0 phenotype, while induced normalised expression was expressed relative to the corresponding basal expression of the untreated flies of the same age. Expression patterns across age groups were analysed with one-way ANOVA followed by SNK comparison tests (summarised in supplementary material Table S2). All analyses were performed using Prism v. 5.01 (GraphPad Software, Inc., San Diego, CA, USA, 2007).

RESULTS

Cold tolerance

Chill coma recovery dynamics were significantly affected by age (χ^2=413.8; P<0.001; Fig. 1A). The fastest recovery was observed in newly emerged flies (age 0), then it increased gradually from day 0 to day 3 phenotypes. A plateau was reached in 3-day-old flies, where recovery dynamics were not further affected by increasing age (Fig. 1A). CT_{min} was also significantly affected by age ($F_{5,138}$=34.01; P<0.001; Fig. 1B). As for chill coma recovery, the lowest values were found in newly emerged flies, and then CT_{min} increased gradually from day 0 to day 3 phenotypes. A plateau was reached in 3-day-old flies, where CT_{min} was not further affected by increasing age (Fig. 1B). Nearly all flies recovered and survived 24 h after the chronic cold stress, so that survival rate ranged between 93 and 98% and was not affected by age (χ^2=3.141; P=0.678) (data not shown).

Stress gene expression

We focused on eight cold-responsive genes and tested whether they exhibited plasticity of expression (induced and basal) according to the age of young flies. Two genes, *hsp23* and *hsp68*, showed significant variation of basal expression according to age (Fig. 2; supplementary material Table S2). A remarkable age-related variation of basal expression was found in these two genes, with 95 and 80% reduction between day 0 and day 5, for *hsp23* and *hsp68*, respectively. Basal expression of the other stress genes (*hsp22*, *hsp40*, *hsp70Aa*, *hsp83*, *Fst* and *Stv*) was not affected by age (Fig. 2; supplementary material Table S2).

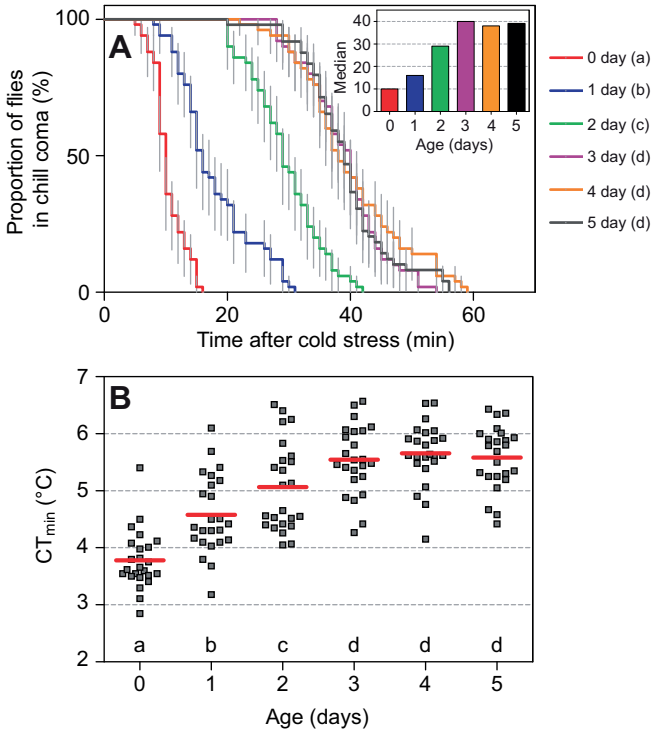

Fig. 1. (A) Temporal recovery curves of *Drosophila melanogaster* females exposed to chronic cold stress (0°C) for 14 h in the six age groups tested (0–5 days). Each point represents the mean proportion (±95% confidence interval) of recovering flies in relation to time after cold stress. Sixty females were tested per age group. The inset shows the median recovery times. Different letters in the key indicate significant differences among recovery curves (Mantel–Cox tests). (B) Critical thermal minimum (CT_{min}) in the six age groups tested. The horizontal lines indicate the mean value (with N=24). Different letters at the bottom indicate significant differences (SNK tests).

Concerning cold-induced expression, we found significant variation according to age in six genes – *hsp22*, *hsp23*, *hsp68*, *hsp70Aa*, *Fst* and *Stv* (Fig. 3, supplementary material Table S2). *hsp22* and *hsp70Aa* had the highest induced expression level in very young phenotypes (day 0 and 1) before declining abruptly in day 2 phenotype. Opposite age-related patterns were found in *hsp23*, *hsp68*, *Fst* and *Stv*, which showed the lowest induced expression levels in the youngest phenotypes (day 0 to 2) before increasing in older groups (Fig. 3).

DISCUSSION

In this study, we investigated intrinsic biological variation of cold tolerance using phenotypes of various ages. In *D. melanogaster*, studies addressing age-dependent stress tolerance have generally used experimental designs where phenotypes of a few days old were compared with older ones (i.e. >30 days) (Sørensen and Loeschcke, 2002). In these cases, a reduction of general stress resistance is expected, as a result of senescence (Minois and Le Bourg, 1999; Grotewiel et al., 2005). However, age-dependent effects unrelated to senescence may also deeply impact on stress tolerance, potentially producing an important source of variation (Bowler and Terblanche,

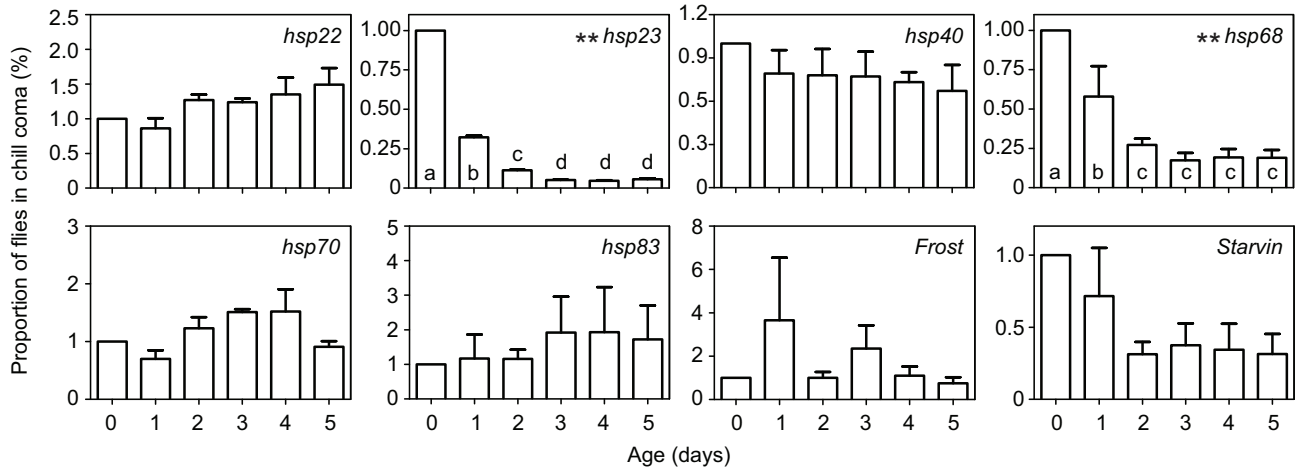

Fig. 2. The mRNA basal level of expression of the assayed genes in the six age groups tested (0–5 days). The basal level (i.e. fold change) of the target gene is expressed relative to day 0 and normalised against the housekeeping reference gene, *RpL13*. Bars represent mean expression (+s.e.m.) derived from three independent biological replicates and three technical replicates. Asterisks indicate when basal expression differs according to age classes (P<0.05) (refer to supplementary material Table S2 for ANOVAs) and, in the case of significance, different letters at the bottom of the bars indicate between-group differences (SNK tests).

2008). Thermal tolerance is a trait that has been much studied in evolutionary and physiological studies (Hoffmann et al., 2003; Angilletta, 2009). Because thermal tolerance is generally assessed in young rather than old phenotypes, age-related variation may confound studies of temperature responses if unaccounted for, an issue not often recognised in experimental design (Pappas et al., 2007; Bowler and Terblanche, 2008). A rapid decline of heat tolerance at young age had previously been reported in holometabolous insects (Bowler, 1967; Bowler and Hollingsworth, 1965; Davison, 1969; Krebs et al., 1998; Sørensen and Loeschcke, 2002; Pappas et al., 2007); however, it was not known whether this within-stage variation was specific to heat tolerance or if it also applied to cold tolerance.

We found that cold tolerance drastically declined at young age. Indeed, both chill coma recovery time and CT_{min} increased with age. David et al. reported that, in *D. melanogaster*, chill coma recovery time globally increased among 3–29-day-old flies (David et al., 1998), but age-dependent cold tolerance variation at young age has not yet been a specific focus of attention. The present results have important implications for future works. Indeed, it would be necessary to take this intrinsic variation into account when designing and performing cold tolerance assays with young insects.

Substantial within-stage variation of heat tolerance occurs in *D. melanogaster*, with embryos being relatively heat-intolerant and pupae and young adults being relatively heat-tolerant (Feder, 1999). Immobile stages, such as larvae and pupae, are especially prone to heat stress because they dwell within necrotic fruits exposed to direct sunlight. By contrast, adults may minimize thermal stress through behavioural compensation and microhabitat selection (Krebs et al., 1998). The ‘developmental carry-over hypothesis’ (Bowler and Terblanche, 2008) suggests that the high heat tolerance of newly emerged adults is a residual attribute of the immobile pupal stadium.

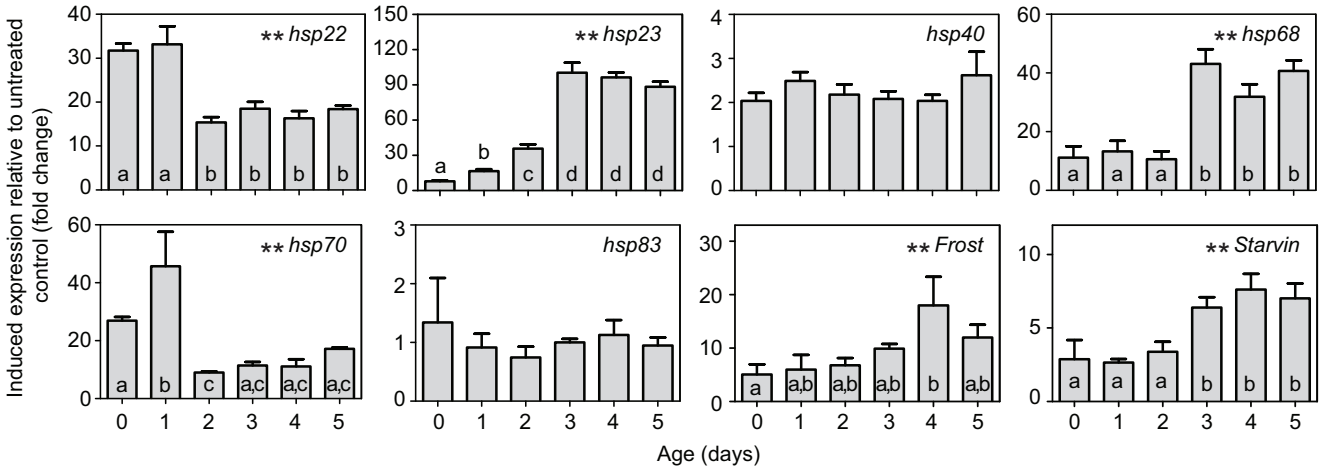

Fig. 3. The mRNA cold-induced level of expression of the assayed genes in the six age groups tested (0–5 days). The expression level was measured in females recovering for 2 h after being exposed to chronic cold stress (0°C for 14 h). The level (i.e. fold change after induction) of the target gene is expressed relative to the control basal level of flies of the same age and normalised against the housekeeping reference gene, *RpL13*. Bars represent mean expression (+s.e.m.) derived from three independent biological replicates and three technical replicates. Asterisks indicate when induced expression differs according to age classes (P<0.05) (refer to supplementary material Table S2 for ANOVAs) and, in case of significance, different letters at the bottom of the bars indicate between-group differences (SNK tests).

The same process seems to apply to cold tolerance. The fact that tolerance to both temperature extremes declines at young age suggests that this response may be part of a general change in environmental stress tolerance.

Conflicting mechanisms may underlie different kinds of stress tolerance; for example, cold tolerance and heat tolerance may entail opposite changes in membrane fluidity (Whitman, 2009). However, adaptation to one extreme does not necessarily cause maladaptation to the other (Angilletta, 2009). Common elements may underlie general mechanisms of stress tolerance (Kültz, 2005). For example, Hsp proteins are key players in conferring tolerance to nearly all kinds of stresses (Sørensen et al., 2003; Whitman, 2009), including heat (Welte et al., 1993; Feder, 1999; Gong and Golic, 2006) and cold stress (Colinet et al., 2010b; Kostál and Tollarova-Borovanska, 2009; Rinehart et al., 2007). Thus, *hsp* genes represent prime candidates for investigating molecular correlates at young age. A previous study (Pappas et al., 2007) found that the basal expression of the protein Hsp70 did not vary according to age, and we corroborate this observation at the transcriptional level. So far, no study has addressed the potential implication of the other members of the heat shock protein family.

Among all stress genes tested, we found that basal expression of *hsp23* and *hsp68* showed a dramatic age-related reduction. In both genes, high basal expression was associated with the most cold-tolerant phenotypes (days 0–1). High constitutive levels of Hsps (genes and proteins) are generally found in organisms living in extreme thermal environments, as found in xeric and polar insect species (Rinehart et al., 2006; Evgen'ev et al., 2007; Clark and Worland, 2008). Although it is not clearly established whether basal levels of Hsps influence, or even set, basal thermal tolerance (Bowler and Terblanche, 2008). Expression of Hsps is known to be modulated during development and metamorphosis, where Hsps serve various functions in protein synthesis and turnover (Mason et al., 1984; Tower, 2011). It has been reported that *hsp23* mRNAs were undetectable in 4-day-old females relative to newly eclosed *D. melanogaster* females, which expressed a high basal level (Mason et al., 1984). Our data corroborate this observation. Moreover, high-throughput expression analyses have also shown a high *hsp23* basal expression in larvae and pupae and a low expression in adults (Graveley et al., 2011). Similarly, in the absence of environmental stress, *hsp68* mRNAs are expressed at a very low level in most developmental stages, but they are at a high concentration in the pupal stage (Mason et al., 1984; Graveley et al., 2011). In addition to their developmental regulation, *hsp23* and *hsp68* are involved in stress tolerance. Increased *hsp23* mRNA levels correlate with increased stress resistance (desiccation, starvation) in selected *D. melanogaster* lines (Kurapati et al., 2000), and knocking down *hsp23* gene expression affects chill coma recovery ability (Colinet et al., 2010b). The gene *hsp68* is closely related to *hsp70* (75% homology) (Palter et al., 1986), and constitutive overexpression of *hsp68* protects flies against oxidative stress and extends lifespan (Wang et al., 2003). At present, it is not known whether there is a genuine causative link between age-related decline in cold tolerance and basal expression of *hsp23* and *hsp68* but there is manifestly a clear-cut association between these patterns. An alternative scenario could be that high basal expression of these *hsp* genes in newly emerged flies might be a developmental carry-over from pupal stage that has no functional link with increased stress tolerance. Clearly, more studies are required to discriminate between these two possibilities.

Concerning induced *hsps* expression, we confirm early observations that the selected genes are upregulated following cold stress, with the exception of *hsp83*, which seems to be slightly cold-responsive but only in males (Colinet et al., 2010a; Colinet and Hoffmann, 2012). In addition, the extent of the upregulations found here match those reported in another study that used strains of different origins (Colinet and Hoffmann, 2012). Significant age-related variations in the amplitude of these upregulations were found in *hsp22*, *hsp23*, *hsp68*, *hsp70Aa*, *Fst* and *Stv*. However, for some genes, the age-related changes occur in different directions. *hsp22* and *hsp70Aa* had their highest cold-induced levels in newly hatched flies, while the opposite pattern was found in *hsp23*, *hsp68*, *Fst* and *Stv*. Two studies have reported that a fall in survival to heat shock at young age correlated with heat-induced Hsp70 expression in *D. melanogaster* (Feder, 1999; Sørensen and Loeschcke, 2002). More recently, Pappas et al. also found a marked reduction in heat-induced Hsp70 expression accompanying the age-related decline of heat tolerance (Pappas et al., 2007). Here, we have demonstrated that a cold induction also results in high *hsp70Aa* mRNA expression at young age. Induced *hsp22* expression also varied with age, with high expression being found in the most cold-tolerant phenotypes. The genes *hsp23*, *hsp68* and *Stv* showed synchronized patterns of induced expression, but in the opposite direction to that of *hsp70Aa* and *hsp22*. The proteins Hsp22 and Hsp23 have different chaperone activity during stress, which suggests different modes of action (Morrow et al., 2006). RNAi directed against *hsp22* and *hsp23* affects chill coma recovery but with different intensities (Colinet et al., 2010b), which suggests that the two genes may contribute to stress tolerance in different ways. The proteins Hsp22 and Hsp23 also differ in subcellular localisation, with Hsp22 being situated in the mitochondrial matrix, whilst Hsp23 is in the cytosol (Michaud et al., 1997; Tower, 2011). The tissue, cell and developmental specificity of expression argue for specialised functions (Michaud et al., 1997), which might explain why *hsp22* and *hsp23* displayed contrasting age-related patterns. The induced expression of *hsp23*, *hsp68* and *Stv* was high in the oldest and least cold-tolerant phenotypes. The protein Hsp68 has a similar protective function to Hsp70, but this function may be part of a temporally different response (Palter et al., 1986). The gene *hsp23* is implicated in stress tolerance, including cold (Kurapati et al., 2000; Colinet et al., 2010a; Colinet et al., 2010b). Stv is a co-chaperone interacting with members of the Hsp70 family and is implicated in stress response (Arndt et al., 2010; Colinet and Hoffmann, 2010). A high induced expression of *hsps* in the least cold-tolerant phenotypes corroborates the suggestion that high inducible Hsps expression does not necessarily reflect a corresponding high level of resistance (or adaptation) but rather that the organisms might be severely stressed (Sørensen, 2010). Hsps are increasingly being implicated in ageing phenotypes (Tower, 2009); however, no senescence effect is expected in flies aged only between 0 and 5 days. A reduced Hsps induction in old cells has been identified and this reduction most likely relates to increased basal levels of Hsps that would inhibit heat-shock response through a feedback loop (Tower, 2009; Tower, 2011). In a similar context, the low induction of *hsp23* and *hsp68* in newly emerged flies might relate to relatively high basal expression in these age groups.

The induced expression of *Fst* (a gene which has no known chaperoning function) also varied with the age of flies. It was previously assumed that *Fst* was a cold-specific gene, as upregulation was not induced by heat shock (Goto, 2001; Sinclair et al., 2007). However, recent studies found that *Fst* mRNAs accumulated during heat exposure (Udaka et al., 2010), and thus *Fst* could play a role in general thermal tolerance. *Fst* has a conserved and consistent molecular role in the recovery from cold stress among *Drosophila* species (Reis et al., 2011; Bing et al., 2012). An RNAi-

based study has shown that *Fst* plays essential roles in recovery from chill coma in *D. melanogaster* (Colinet et al., 2010c). In addition, a significant association between Fst allele size (PEST region) and chill coma recovery was found in *Drosophila americana*, which further confirms a relationship between *Fst* and chill coma recovery (Reis et al., 2011).

In the present study, we only tested females. The patterns of Hsps expression are sex-specific (Mason et al., 1984; Sørensen et al., 2007); therefore, the molecular stress responses might differ between the sexes. Further experiments would be necessary to verify whether the patterns observed in females differ from those of males. The molecular mechanisms underlying the within-stage variation of stress tolerance at young age are still unknown, but our candidate gene approach has shown that transcriptional regulation of some stress genes is associated with this variation. High basal expression of *hsp23* and *hsp68* may provide supporting evidence for the involvement of constitutively expressed *hsps* in setting basal thermal tolerance. Alternatively, a developmental carry-over from high expression of these genes in the pupal stage might be considered. The functional and evolutionary significance of variation in the expression of Hsps likely depends on the interaction of developmental stage and probability of stress exposure (Feder, 1999). This study offers fertile ground for further studies in order to clarify whether the associations found here imply real causations. In addition, the temporal dynamics and amplitude of the expression might differ between *hsp* mRNAs and Hsp proteins (Bahrndorff et al., 2009), so that it will be useful to validate our results at the protein level. Finally, one should bear in mind that many genes and traits may affect variation in thermotolerance, of which the expression of *hsps* is only one. Therefore it will be useful to study other genes and molecular pathways to understand the underpinnings of stress tolerance variation at young age.

Overall, the present study shows that, similar to heat tolerance, cold tolerance declines dramatically from the onset of the adult stage in *D. melanogaster*. This observation suggests that the general thermal tolerance is affected. The biological and evolutionary meaning of such intraspecific stress tolerance variation is at present unknown. Contrary to immobile stages, adults can use behavioural avoidance of thermal and other stresses. Therefore, once sexual maturity is reached, fitness might be optimized by investing more resources on reproduction than protection (Sørensen and Loeschcke, 2002). Without determining the intraspecific factors affecting thermal tolerance, the understanding of how temperature sets mortality of ectotherms, and hence their population dynamics and biogeography, will remain equivocal (Bowler and Terblanche, 2008). Future studies will have to consider this within-stage variation when determining thermotolerance of young phenotypes.

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