Journal of Insect Physiology 71 (2014) 156–163

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Analysis of survival, gene expression and behavior following chill-coma in the medfly *Ceratitis capitata*: Effects of population heterogeneity and age

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ARTICLE INFO

Article history:
Received 29 May 2014
Received in revised form 2 October 2014
Accepted 28 October 2014
Available online 4 November 2014

Keywords:
Ceratitis capitata
Chill-coma recovery
Chilling-injury
Survival
Population heterogeneity
Superoxide dismutase

ABSTRACT

The medfly *Ceratitis capitata* is an agricultural pest distributed worldwide thanks, in part, to its phenotypic plasticity of thermal tolerance. Cold exposure has been shown to reduce *C. capitata* survival, which may affect its distribution in areas with subfreezing temperatures. When insects are increasingly cooled, they attain a critical thermal threshold and enter a chill-coma state characterized by cessation of movement. It is not clear how a rapid cold exposure affects the physiological state of medflies, and how this is influenced by age and population heterogeneity. In order to approach these questions, *C. capitata* single-sex laboratory populations of 15 and 30 days old were subjected to a chill-coma recovery assay, and separated according to their recovery time in three subgroups: Fast-Subgroups, Intermediate-Subgroups, and Slow-Subgroups. Thereafter, we analyzed their survival, behavioral, and gene expression outputs. In female and old male populations, we found that flies with the slowest recovery time had a reduced life expectancy, a higher initial mortality rate, and a worse climbing performance compared with flies that recovered faster. Therefore, we were able to separate subgroups that developed chilling-injury from subgroups that had a reversible full recovery after cold exposure. The gene expression analysis of the heat shock protein genes *hsp70* and *hsp83* showed no clear association with the parameters studied. Interestingly, thorax expression levels of the Cu/Zn superoxide dismutase gene were elevated during the recovery phase in the Fast-Subgroups, but remained constant in the Slow-Subgroups that developed chilling-injury. On the other hand, none of the young male subgroups seemed to have suffered irreversible damage. Thus, we concluded that depending on age and population heterogeneity, chill-coma recovery time points out significant differences on individual cold tolerance. Moreover, the inability to properly induce the antioxidant defense system to counteract the oxidative damage caused by cold seems to contribute to the development of chilling-injury.

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1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* is a worldwide pest of great economic impact, used as a model in demographic senescence studies (Carey, 1997, 2011). It is important to understand the factors that determine its distribution and abundance and, in this regard, the ability of *C. capitata* to adjust to temperature variations has been recently investigated, showing a broad thermal tolerance (Nyamukondiwa and Terblanche, 2009; Nyamukondiwa et al., 2010; Weldon et al., 2011; Basson et al., 2012). Basson et al. (2012) demonstrated that cold exposure reduced *C. capitata* survival, which suggests the possibility of a chill-dependent injury, although flies showed no metabolic or fecundity costs. Therefore, it would be interesting to investigate the effects that cold exposure produce in the physiological state of *C. capitata* individual flies within a population, and how this affects their survival and distribution.

Insects have specific strategies against temperature variations, including behavioral, physiological, and biochemical adaptations

Abbreviations: CCR, chill-coma recovery; Hsp, heat shock protein; Sod, Cu/Zn superoxide dismutase; FSG, Fast-Subgroup; ISG, Intermediate-Subgroup; SSG, Slow-Subgroup.

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http://dx.doi.org/10.1016/j.jinsphys.2014.10.015
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(Denlinger and Lee, 1998; Denlinger and Yocum, 1998). Upon a rapid decrease in temperature, biological membranes are affected, resulting in intracellular organelle damage and dissipation of ions and other solutes (Colinet et al., 2010). Thus, one strategy to maintain the fluidity of membranes is to increase the proportion of polyunsaturated fatty acids in membrane phospholipids (Overgaard et al., 2005). Other factors involved in cold tolerance are the generation of cryoprotectants, such as glycerol and trehalose, the production of ice nucleators, and the regulation of water content (Clark and Worland, 2008). At a gene expression level, cold induces the expression of genes involved in circadian rhythm, metabolism, and heat shock response, such as Heat shock proteins (Hsps), and other specific cold response genes, such as *Starvin* and *Frost* in *Drosophila* (Sinclair et al., 2007; Colinet et al., 2010, 2013; Vesala et al., 2012).

When insects are increasingly cooled, they reach a critical thermal threshold and enter in a chill-coma, a state of reversible cold anesthesia. This cessation of neuromuscular activity has been attributed to failure on Na^+/K^+-ATPase pumps, which decreases ion gradients and transmembrane voltages in nerve cells (Hosler et al., 2000; Koštál et al., 2006). Even in the absence of freezing, increasing levels of cold may also cause chilling-injury, which produces an irreversible fitness decrease, and even death (Macmillan and Sinclair, 2011a). Chilling-injury that occurs within minutes to hours after rapid cooling has been attributed to loss of membrane integrity due to phase transitions in lipid membranes (Lee, 1989). Moreover, when injury occurs to the mitochondrial membrane and the electron transport system is affected, there may be a greater production of reactive oxygen species. Thus, oxidative stress is thought to contribute to insect tissue damage after cold exposure (Denlinger and Lee, 1998). In this regard, Rojas and Leopold (1996) demonstrated that long-term exposure of houseflies to 7 °C produces chilling-injury, which correlates with the activity of the antioxidant enzyme superoxide dismutase (SOD). In the oriental fruit fly, *Bactrocera dorsalis*, exposure of flies to zero and sub-zero temperatures during 3–9 h periods also resulted in an increase in SOD activity after 30 min of recovery at 27 °C (Jia et al., 2011).

We think that individual variation within a population (i.e., population heterogeneity) may influence the proportion of individuals that develop chilling-injury during a cold exposure. Even in genetically homogeneous strains of insects, there is heterogeneity among individuals in a population attributed to random variations in the developmental and breeding microenvironments (Khazaeli et al., 1995). Moreover, some subpopulations may have a low fitness under specific conditions but a better performance in other environments (Romanyukha et al., 2010). Stress experiments are useful to recognize individual variation, provided that the physiological status of individuals who survive the different levels of stress is took into consideration (Curtsinger and Khazaeli, 1997). Chill-coma recovery (CCR) time has been used in flies to measure the ability of individuals to become active after being knocked down by a cold stress (David et al., 1998; Macdonald et al., 2004), and it may be a plausible variable to study individual variation. CCR time increases in *Drosophila melanogaster* with age (David et al., 1998) and, together with negative geotaxis, has been defined as a health measure during infection in this species (Linderman et al., 2012).

The aim of our study was to better understand the effects of cold exposure in the physiological state and survival of *C. capitata* laboratory populations, and how this may be influenced by individual variation, age and sex. We hypothesized that the recovery time of flies after chill-coma can be used to separate subgroups within a population with differences in their cold tolerance, and therefore in their susceptibility to develop chilling-injury. We further hypothesized that differences in the expression of stress-related genes may come along with the development of chilling-injury, and the subsequent reduction in survival and behavioral performance. To address our hypotheses, we separated young (15 days old) and old (30 days old) single-sex experimental populations into subgroups according to their CCR time. Our first specific objective was to characterize subgroups in medfly populations with different cold tolerance, analyzing their survival parameters and negative geotaxis performance. This behavioral assay reflects the locomotor ability of flies to respond to a mechanical stimulus (Gargano et al., 2005; Riemensperger et al., 2013), and was useful to corroborate differences between flies with reversible and irreversible damage after chill-coma, since the neuromuscular activity is affected by cold exposure. The second objective was to find a possible association between these parameters and the cold stress response at a gene expression level. We focused on the expression of *hsp83* and *hsp70*, previously characterized in *C. capitata* (Papadimitriou et al., 1998; Theodoraki and Mintzas, 2006) and associated with cold response and aging in other insects (Yocum et al., 1991; Sinclair et al., 2007; Udaka et al., 2010; Tower, 2011), and on the Cu/Zn superoxide dismutase (*sod*).

2. Methods

2.1. Fly rearing

A well-established laboratory *C. capitata* wild-type strain, Mendoza, was used for this study, and maintained as previously described (Pujol-Lereis et al., 2012). This strain was originally isolated as a colony of several individuals from a very polymorphic Argentinean population (Basso et al., 2009), and after about 300 generations, a significant degree of genetic heterogeneity still persists in our laboratory population. For the experiments, virgin adult flies were collected less than 12 h after emergence from the puparium, sexed under CO_2 anesthesia, and placed in 3.75 L flasks with free access to sucrose: dry yeast (3:1) and 1% agar as sources of food and water, respectively. Flies were kept on a 16:8 h light:dark cycle at 23 °C. These single-sex experimental populations consisted at day 1 (day of emergence) of 100 virgin flies. Food and water were renewed every 5 days.

2.2. Chill-coma recovery (CCR) assay and determination of subgroups

Single-sex experimental populations (100 flies at day 1, see Section 2.1) were maintained until days 5, 15 or 30, and subjected to CCR assay. For this purpose, flies were placed in Petri dishes and cooled to 0 °C on ice. After 4 h, flies were moved back to 23 °C and placed in ventral position. Recovery time was recorded. A fly was considered recovered when able to stand again on its legs. For determination of subgroups, flies were individualized as soon as they had recovered, and the CCR time of each fly was recorded (flies that recovered at the same time were put together). This procedure was not possible with 5 days old populations, because flies recovered almost altogether in a short period of time (Fig. 1). Populations of 15 and 30 days of age were separated in three subgroups, with a fast, intermediate and slow recovery time: Fast-Subgroup (FSG), Intermediate-Subgroup (ISG) and Slow-Subgroup (SSG), respectively. The mean and variance of CCR time varied depending on experimental population (i.e., replicate), age and sex. Therefore, it was not possible to establish exact recovery time values as thresholds among the subgroups. From each experimental population, the flies that recovered faster and most of them one at a time, were grouped in the FSG. After a while, groups of three or more flies started recovering together, and were grouped in the ISG, which corresponded to the average of the population. After most of the flies had recovered, there were flies with a higher CCR time that recovered also one at a time, and so grouped

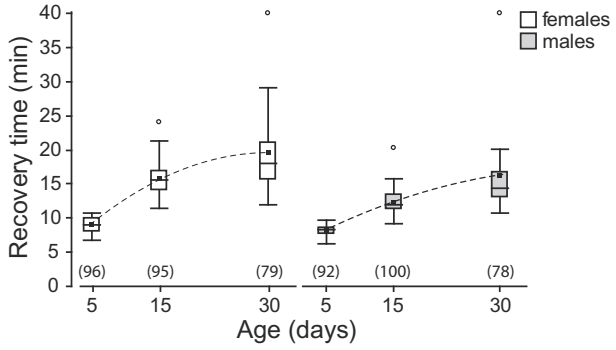

Fig. 1. Chill-coma recovery time of *C. capitata* populations increase with age. Boxplots of the recovery time (min) of female (white) and male (gray) populations at 5, 15 and 30 days of age. The upper and lower ends of the center box indicate the 75th and 25th percentiles, respectively. The line inside the box indicates the median and the bars indicate 1.5 times the distance of the interquartile range from the median. Black dots mark the mean, and white circles mark potential outliers (more than 3 S.D. from the mean). Numbers in brackets indicate the total number of individuals. Data were fitted to polynomial regressions (dashed lines; $p < 0.001$).

in the SSG. As an example, the percentage of individuals in the subgroups used for longevity measurements is shown in Table 1. For behavioral and expression experiments, these proportions were similar. Flies that never recovered from chill-coma, although this was unusual, were not used in the experiments.

2.3. Negative geotaxis assay

Rapid iterative negative geotaxis (RING) assays were performed according to Gargano et al. (2005) and adapted to medfly as previously described (Pujol-Lereis et al., 2012). Trials were started after 5 h of recovery from chill-coma, at 23 °C. Briefly, a sample of 10 flies from each subgroup was transferred to a 250 mL test tube. Flies were forced to the bottom of the test tubes by gentle tapping, and their position 10 s later was recorded with a digital camera (Sony DSC-W100). The distance climbed by the flies was measured using the Scion Image 4.0 software (Scion Corporation). Three independent replicates were analyzed for each group.

2.4. Survival experiments

Single-sex experimental populations were subjected to CCR assay at 15 or 30 days of age, and separated into subgroups. Thereafter, flies were maintained under the same conditions as prior to the CCR assay (see Section 2.1), and followed over time. Dead flies were daily counted and removed. Nine independent replicates were analyzed for each group.

2.5. Gene expression (RT-PCR)

For gene expression studies, samples of 5 flies were taken from each experimental population before the exposure to cold and during the exposure phase (2 h at 0 °C), and immediately dissected. Then, populations were separated in subgroups, and samples of 10 flies from FSG and SSG were dissected 2 h after the shift to 23 °C. Total RNA was isolated from head and thorax using TRI Reagent kit (Sigma–Aldrich Co., MO, USA). cDNA was prepared from total RNA (0.5 μg) by reverse transcription using Oligo-dT and M-MLVRT (Moloney Murine Leukemia Virus Reverse Transcriptase) (Promega, WI, USA), according to the instructions of the manufacturer. Semiquantitative RT-PCR were performed using the following primers: *hsp70Fw* AAGGAAATGAGTTCGGGCAATGCC, *hsp70Rv* TGAATCCGCCAGCCTGTTGACA; *hsp83Fw* GTCTGAAGAAGTGGAAACCTTCG, *hsp83Rv* CCATGAATGCTTTAGTGCCGG; *sodFw* TGGTGGTAAAAGCTGTATGCG, *sodRv* GCAAATGACGCCAC; *actinFw* ACGGCATCATCACCAACTG, *actinRv* TACCGCATGATTCCATGCCC; *18SFw* GGTTCGAAGGCGATCAGATA, *18SRv* TTCCGCAGGTTCACCTACG. The products obtained were then analyzed on 1% agarose gels stained with ethidium bromide. Quantification was performed with the Image J software (http://imagej.nih.gov/ij/). Since *actin* expression levels were too variable among treatments, the expression levels of each gene were normalized to *18S rRNA* levels. Three independent replicates were analyzed for each group.

2.6. Statistical analyses

Gompertz mortality model was fitted to mortality data using the software WinModest Version 1.0.2 (Pletcher, 1999). For each subgroup, we calculated the mortality parameters of the Gompertz model ($\mu_x = ae^{bx}$), where μ_x is the age-specific mortality at age x, a is the initial mortality rate, and b is the age-dependent increase in mortality rate (slope parameter). The mortality parameters estimated for each subgroup were compared by likelihood ratio tests (Pletcher, 1999), and a Bonferroni correction was applied.

Analyses of variance (ANOVAs) were done using the Infostat 2010 Software (UNC, Córdoba, Argentina). Blocked ANOVAs were carried out when necessary.

Table 1
Survival parameters of *C. capitata* subgroups separated by CCR assays.

	Subgroup[a]	*N* (%)[b]	Life expectancy (days ± S.E.M.)	Maximum lifespan	Initial mortality rate (*a*)	Slope parameter (*b*)
Females 15 days	FSG	156 (21.1)	48.5 ± 2.6[a]	112	0.014[a]	0.0247[a]
	ISG	426 (57.6)	44.0 ± 1.5[a,b]	130	0.024[b]	0.0140[b]
	SSG	158 (21.3)	38.1 ± 1.8[b]	112	0.036[c]	0.0085[b]
	no CC	234 (100)	40.8 ± 2.5	139		
Females 30 days	FSG	169 (29.0)	50.3 ± 2.0[a]	116	0.052[a]	0.0132
	ISG	267 (45.4)	48.4 ± 1.6[a]	121	0.060[a]	0.0092
	SSG	147 (25.2)	41.4 ± 0.6[b]	68	0.122[b]	0.0090
	no CC	161 (100)	49.9 ± 1.1	139		
Males 15 days	FSG	205 (26.8)	34.5 ± 3.5	126	0.036	0.0154
	ISG	420 (54.8)	34.4 ± 3.7	103	0.038	0.0145
	SSG	141 (18.4)	35.4 ± 3.9	82	0.031	0.0229
	no CC	273 (100)	33.8 ± 0.2	86		
Males 30 days	FSG	146 (34.0)	41.9 ± 1.4[a]	101	0.061[a]	0.0159
	ISG	161 (37.5)	40.2 ± 0.8[a]	107	0.082[a]	0.0104
	SSG	123 (28.5)	35.9 ± 0.6[b]	54	0.156[b]	0.0140
	no CC	161 (100)	41.2 ± 0.4	86		

Different letters indicate significant differences among subgroups ($p < 0.05$).

[a] FSG, Fast-Subgroup; ISG, Intermediate-Subgroup; SSG, Slow-Subgroup; no CC: populations maintained at 23 °C and not subjected to chill-coma (not included in statistical analysis).

[b] Total number of individuals and the percentage of the population in brackets. For subgroups, data from nine populations were pooled per sex and age.

Curve fittings (polynomial regressions) were done using Origin 8.5 (OriginLab, Northampton, MA, USA).

3. Results

3.1. Chill-coma recovery time and determination of subgroups

C. capitata single-sex populations maintained at 23 °C were subjected to a CCR assay at 5, 15 and 30 days of age. As shown in Fig. 1, the recovery time of the populations increased with age for both sexes, and seems to be more variable among female flies compared with males.

Then, CCR assays were used to separate 15 and 30 days old experimental populations into three recovery-dependent subgroups: Fast-Subgroup (FSG), Intermediate-Subgroup (ISG), and Slow-Subgroup (SSG) (see Section 2.2). Fig. 2A–D shows the recovery time of subgroups according to sex and age. The boxplot graphs show that the variability in recovery time of flies was greater for SSG, except for 15-day-old males.

3.2. Behavioral characterization of the subgroups

We carried out a rapid iterative negative geotaxis assay (RING) to test the locomotor ability of flies from the different subgroups. We observed that flies from SSG climbed a lower distance comparing with FSG and ISG in females separated at 15 ($p = 0.02$) and 30 ($p < 0.001$) days of age (Fig. 2E–F), and in males separated at 30 days ($p = 0.001$; Fig. 2H). Thus, these subgroups differed not only in their recovery from chill-coma, but also in an evoked locomotor response, suggesting the development of chilling-injury in SSG. For males separated at 15 days of age, no differences were observed in negative geotaxis performance among the subgroups (Fig. 2G).

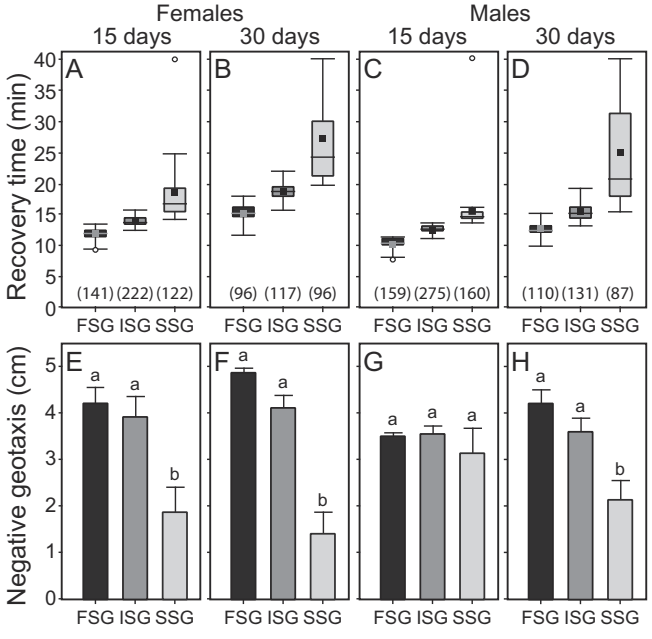

Fig. 2. Chill-coma recovery time and behavioral output of *C. capitata* subgroups. Subgroups separated at 15 or 30 days of age: FSG, Fast-Subgroup (black); ISG, Intermediate-Subgroup (dark gray); SSG, Slow-Subgroup (light gray). (A–D) Boxplots of the recovery time (min) of the subgroups (see boxplot explanation in Fig. 1). Values from three independent populations were pooled per sex and age. Numbers in brackets indicate the total number of individuals. (E–H) Negative geotaxis performance (cm) of the subgroups 5 h after the shift to 23 °C. Each value corresponds to the mean of three replicates (±S.E.M.; 10 flies per replicate). Statistics: different letters indicate significant differences among subgroups ($p < 0.05$; one-way blocked ANOVA followed by Tukey's test).

3.3. Survival curves and life expectancy

Survival curves were found to be distinct among 15-day-old female subgroups (Fig. 3A), and also 30-day-old male subgroups (Fig. 3D). For 30-day-old females, FSG and ISG presented superposed curves that were different from SSG (Fig. 3B). In accordance to these results, the remaining life expectancy of FSG was higher than SSG in females separated at both ages (ANOVA: 15 days, $p = 0.002$; 30 days, $p < 0.001$) and in males separated at 30 days of age (ANOVA: $p < 0.001$), while differences were not significant between FSG and ISG (Table 1). On the other hand, the shape of the survival curves and the remaining life expectancies were not different among 15-day-old male subgroups (Fig. 3C and Table 1). Although maximum lifespans of 15-day-old male subgroups were different (Table 1), we considered life expectancy as a much better descriptor of survival than maximum lifespan, which is determined by a single long-lived fly.

Populations not subjected to the acute stress of a chill-coma (Pujol-Lereis et al., 2012) showed similar life expectancy than the stressed subgroups at 15 days of age (Table 1). For 30-day-old flies, non-stressed flies have similar life expectancy than FSG and ISG, but it seems higher than SSG (Table 1). This fact points to the possibility of an essential difference in the physiological status of insects in the SSG.

3.4. Mortality rates of subgroups

To better understand the demographic differences among the subgroups, we evaluated Gompertz mortality rates (see Section 2.6). In 15-day-old females, the initial mortality rate was higher in FSG than in SSG ($p = 0.001$), having ISG an intermediate value (ISG vs. FSG, $p = 0.007$; ISG vs. SSG, $p = 0.03$), while the slope parameter was higher in FSG than ISG ($p = 0.04$) and SSG ($p = 0.01$) (Table 1). For both sexes, 30-day-old SSG showed higher initial mortality rates than FSG (females and males, $p < 0.001$) and ISG (females, $p < 0.001$; males, $p = 0.001$), but no differences in the slope parameter (Table 1). In agreement with negative geotaxis

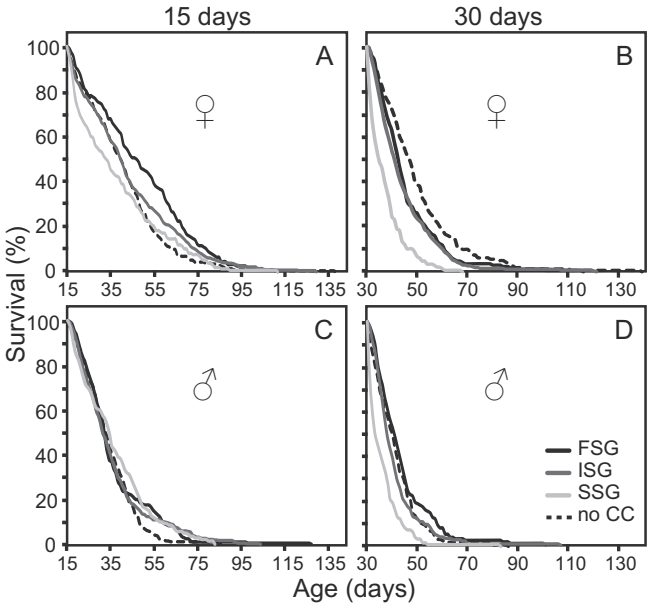

Fig. 3. Survival curves of *C. capitata* subgroups. Percentage of survival of female (A and B) and male (C and D) subgroups separated at 15 or 30 days of age. FSG, Fast-Subgroup (black lines); ISG, Intermediate-Subgroup (dark gray lines); SSG, Slow-Subgroup (light gray lines); no CC: populations maintained at 23 °C and not subjected to chill-coma (black dashed lines) (Pujol-Lereis et al., 2012).

performance and life expectancy, subgroups of 15-day-old males showed no differences in their mortality parameters (Table 1).

3.5. Chill-coma induced stress response

To explore the stress response of the subgroups, we analyzed possible differences at gene expression level of two Hsps, *hsp70* and *hsp83* (Hsp90 gene in flies), and of the oxidative stress related protein Cu/Zn superoxide dismutase, *sod*.

Regarding age-dependent changes, we observed an increase in basal *hsp83* levels in female thorax and in *hsp70* levels in males' head and thorax with age (Fig. 4A, D and E), and also a decrease in basal *sod* levels in thorax of both sexes (Fig. 4F). No differences were observed in *hsps* levels between flies non-exposed to chill coma (0 h exposure) and during the exposure phase (2 h exposure) (Fig. 4A, B, D and E). However, we observed a decrease in *sod* levels during the exposure phase (Fig. 4C and F).

We then evaluated if expression levels after chill-coma differed among the subgroups. As *sod* levels decreased in cold exposed flies (Fig. 4), we compared expression levels during the exposure phase (2 h exposure), and 2 h after the shift to 23 °C for FSG and SSG (see Section 2.5).

In heads (mostly brain tissue) of both sexes and ages, *hsp70* and *hsp83* expression increased during the recovery phase in FSG and SSG compared with the exposure phase, but no differences were observed between subgroups (Fig. 5A and B). *sod* expression in heads remained constant after 2 h of recovery for both subgroups (Fig. 5C).

In thorax (mostly muscle tissue), *hsp70* levels increased during the recovery phase in FSG and SSG compared with the exposure phase for both sexes and ages (Fig. 5D). No significant differences were detected between subgroups, although males *hsp70* levels in SSG were slightly lower than in FSG (Fig. 5D). *hsp83* levels were also increased during the recovery from chill-coma (Fig. 5E). In 15-day-old females and males, the expression of *hsp83* was significantly lower in SSG than in FSG, whereas at 30 days of age there were no significant differences between subgroups (Fig. 5E). On the other hand, *sod* levels in the thorax of females at both ages and males at 30 days old increased significantly for FSG, but did not change for SSG in comparison with the exposure phase (Fig. 5F). However, *sod* levels in 15-day-old males remained constant after 2 h of recovery from chill-coma (Fig. 5F).

4. Discussion

C. capitata, which has probably originated in East Africa (Baliraine et al., 2004), has successfully invaded many countries worldwide (Malacrida et al., 2007), a fact mainly explained by

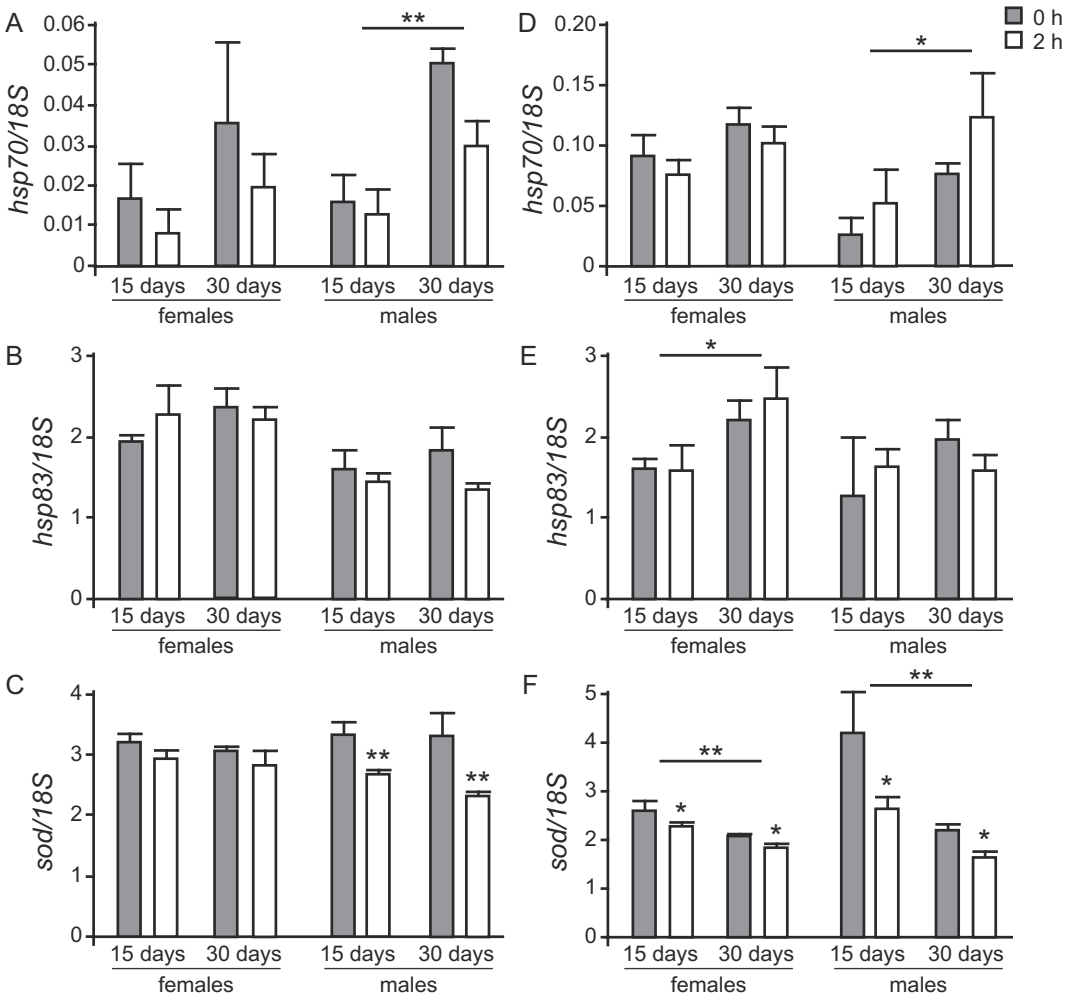

Fig. 4. Gene expression levels of flies non-exposed and during exposure to 0 °C. mRNA levels of *hsp70* (Heat shock protein 70; A and D), *hsp83* (Heat shock protein 83; B and E), and *sod* (Cu/Zn superoxide dismutase; C and F) in head (A–C) and thorax (D–F) of flies non-exposed to chill-coma (0 h, gray bars) and during the exposure phase (2 h, white bars). Relation between the levels of the mRNA of interest and the ribosomal RNA 18S in females and males at 15 and 30 days of age. Each value corresponds to the mean of three replicates (±S.E.M., 5 flies per replicate). Statistics: a two-way ANOVA was carried out for each sex, body part and gene; $^{*}p < 0.05$, $^{**}p < 0.01$.

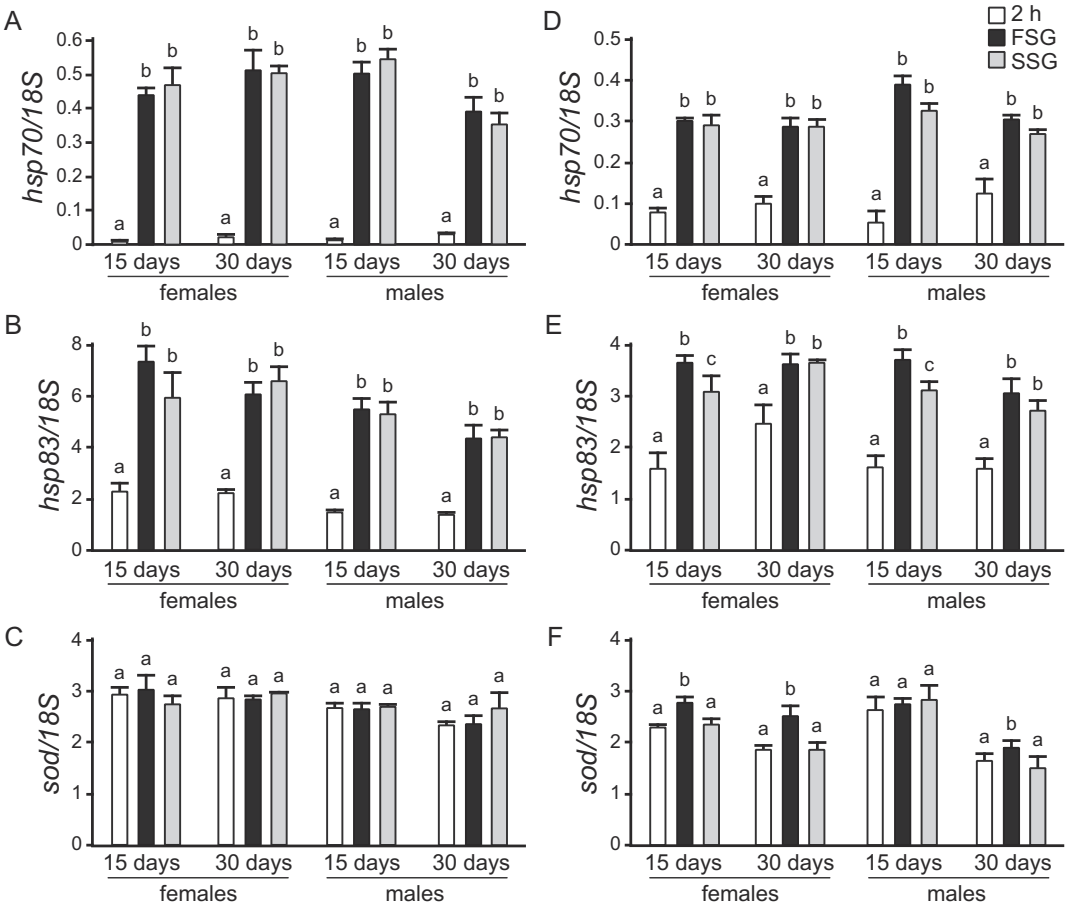

Fig. 5. Gene expression levels of subgroups. mRNA levels of *hsp70* (Heat shock protein 70; A and D), *hsp83* (Heat shock protein 83; B and E), and *sod* (Cu/Zn superoxide dismutase; C and F) in head (A–C) and thorax (D–F) of flies during the exposure phase, and 2 h after the shift to 23 °C (subgroups FSG and SSG). Relation between the levels of the mRNA of interest and the ribosomal RNA 18S in females and males at 15 and 30 days of age. Flies during exposure phase (2 h, white bars); FSG, Fast-Subgroup (black bars); SSG, Slow-Subgroup (light gray bars). Each value corresponds to the mean of three replicates (±S.E.M.; 5 flies per replicate for the 2 h exposure groups; 10 flies per replicate for FSG and SSG). Statistics: different letters indicate significant differences among groups ($p < 0.05$; one-way blocked ANOVA followed by Duncan's test).

multiple introductions that allowed maintenance of genetic variability, the wide variety of host plants (Liquido et al., 1991), and variation in physiological tolerance to climatic stress (Nyamukondiwa et al., 2010). The presence of *C. capitata* in areas with subfreezing temperatures during winter has been suggested to be a consequence of migration from nearby temperature-favorable areas at the beginning of the summer, rather than overwintering of pre-imagos or adults (Israely et al., 2004). In order to further investigate the physiological tolerance and survival of *C. capitata* after a rapid cold exposure, we applied a chilling stress to medfly laboratory populations at 5, 15 and 30 days of age. Here, we showed that CCR time increased with age for both sexes, and that females had higher mean and variability of CCR time than males. Nyamukondiwa and Terblanche (2009) measured maximum and minimum critical thermal limits, i.e., the cooling temperature at which insects lose coordinated muscle function, and showed that 28-day-old medflies generally had poorer thermal tolerance than younger flies, which is consistent with our results, although they found no influence of gender. Males and females may behave similarly regarding certain aspects of thermal tolerance such as critical thermal limits, and differently in others such as CCR time. We also observed an increase in the degree of CCR time variability with age that reveals the heterogeneity among individuals in their cold tolerance, and may be a consequence of changes in the composition of individuals in the population over time (Roach and Gampe, 2004). Individual variation within a population, or heterogeneity, suggests that individuals differ in unobserved susceptibility to death due to random developmental and environmental variation (Wu et al., 2006), even in genetically homogeneous strains of insects such as laboratory populations (Khazaeli et al., 1995). This variation may be responsible of the different ability of individuals in a population to resist stress conditions (Vaupel et al., 1979).

In order to study population heterogeneity in regard to cold tolerance, subgroups were obtained from young (15 days) and old (30 days) experimental populations according to their CCR time. We found that slow-recovery subgroups with a high variability presented lower climbing performance and life expectancies, and higher initial mortality rates, which suggests that these flies had suffered irreversible chilling-injury. This agrees with results in *Drosophila* suggesting that slow and highly variable recovery from chill-coma is indicative of injury (Macmillan and Sinclair, 2011a). Our results demonstrated that CCR time was useful to discriminate between flies that experienced a full reversible recovery from chill-coma, and flies that developed chilling-injury.

To elucidate possible differences between reversible and irreversible physiological changes after cold at a gene expression level, we measured the expression of stress-related genes *hsp70*, *hsp83* and *sod*. Regarding age-dependent changes, we observed an increase in basal *hsp83* levels in female thorax, which were not seen in whole body *C. capitata* analyses by other authors (Theodoraki and Mintzas, 2006), and an increase in *hsp70* levels in males' head and thorax with age. We also observed a decrease

in basal *sod* levels in thorax, but not in head, suggesting a higher susceptibility of muscles to oxidative damage with age. No differences were observed between non-exposed (0 h) and during exposure (2 h) *hsps* levels, as previously reported in *D. melanogaster* for *hsps*, *Frost* and *Desaturase2* (Sinclair et al., 2007; Colinet et al., 2010). However, we observed a decrease in *sod* levels during the exposure phase, demonstrating a different regulation of *sod* expression under cold stress in *C. capitata*.

When analyzing subgroups, the expression levels of *hsps* were associated with life expectancy only in young females, in which FSG had higher life expectancy and *hsp83* levels than SSG. These results are in accordance with studies by Wu et al. (2006) which showed that *Caenorhabditis elegans* worms with higher *hsp16.2* levels had a longer lifespan. However, our young male *C. capitata* subgroups showed differences in *hsp83* expression but similar life expectancies. Moreover, differences in life expectancies in subgroups separated at older ages were not accompanied by differences in *hsps* levels. In *D. melanogaster*, *hsp70* and *hsp22* transgene expression in young flies was only partially predictive of their remaining lifespan under normal and stress conditions (Yang and Tower, 2009). Thus, *hsps* expression is not straightly correlated with survival and chilling-injury, and, as it has been suggested by Sørensen (2010), the level of induction of *hsps* expression not necessarily reflect the level of resistance or adaptation to stress. Measurement of expression levels of other Hsp genes in *C. capitata*, such as *hsp23* and *hsp27* (Kokolakis et al., 2008, 2009), may contribute to the understanding of cold response after chill-coma.

Interestingly, FSG of females at both ages and 30-day-old males showed higher thorax *sod* levels than SSG. This increase in FSG *sod* levels during the recovery phase is consistent with previously reported increases in SOD enzymatic activity in the oriental fruit fly, *B. dorsalis* (Jia et al., 2011), and in the beetle *Alphitobius diaperinus* (Lalouette et al., 2011). Therefore, we demonstrated that subgroups of flies that developed chilling-injury had lower *sod* levels than subgroups with the same age and gender that experienced a reversible chill-coma. Studies in null mutants of *C. elegans* suggested that SOD activity does not influence longevity under normal conditions, but affects survival after a stressful episode, including osmotic, cold and heat stress (Van Raamsdonk and Hekimi, 2012). It might be possible that medfly individuals failing to increase *sod* expression during the first hours of recovery from chill-coma are more affected by oxidative damage and therefore develop chilling-injury. Behavioral performance, life expectancy and mortality rates were not different among 15-day-old male subgroups, showing that young males were somehow resilient to permanent chilling-injury in our experimental conditions. Moreover, *sod* thorax expression of 15-day-old males remained constant after chill-coma, and basal levels were higher than in 30-day-old males. Rojas and Leopold (1996) showed for developmental stages of the house fly that SOD activity was unchanged in the more cold-resistant stage after 7 days at 7 °C, which suggest a sufficient SOD activity to overcome oxidative species. We then conclude that at least one of the reasons why 15-day-old males are more cold tolerant than older 30-day-old males is that their higher basal *sod* expression levels can better counteract oxidative damage.

Differences among subgroups were more pronounced in the thorax (mostly flight muscles) rather than in the head (mostly central nervous system tissue), suggesting that medfly muscles are more sensitive to low temperature than the nervous system. As far as we know, differences in the resilience to cold between insect neural and muscle tissues have not been reported. As already mentioned, disturbance of ion homeostasis has been suggested to be one of the main causes of chilling-injury (Zachariassen et al., 2004). Although this loss of homeostasis is involved in loss of muscle excitability, brain and ventral ganglia nerves are protected by the blood–brain barrier from direct changes of solutes with the hemolymph, and therefore from systemic disturbances of ion homeostasis (MacMillan and Sinclair, 2011b). This may be a possible reason why no differences in the expression of stress-related genes measured in this study were seen in the head between subgroups. Although there is an increase in *hsps* expression levels during the recovery phase, there are no changes in *sod* expression levels in the head, which suggests that the damage experienced by the brain is not sufficient to induce the antioxidant defense system. We have previously demonstrated for *C. capitata* that a mild heat stress produce greater changes in head main lipid profiles than in thorax (Pujol-Lereis et al., 2012). Lipid profiles of chill-coma subgroups will be useful to better understand differences in the susceptibility of tissues to chilling-injury.

Bowler and Terblanche (2008) pointed out the importance of taking into account the age of individuals in thermal biology studies not only as a source of variation, but also as a tool to test aging theories. The biodemographer Vaupel (2010) hypothesized that at least at advanced ages, individuals deteriorate at the same rate and, therefore, subpopulations that differ in their level of mortality have the same rate of increase in mortality with age. In agreement with this hypothesis, we showed that 30-day-old subgroups had different initial mortality rates (parameter *a*), and the same slope of increase in mortality (parameter *b*). Therefore, it would be interesting to further investigate the deterioration rate of individuals in the CCR subgroups, which may contribute to the field of aging research.

Overall, we corroborated our hypothesis that CCR time is a valuable tool to separate individuals in a population that have developed chilling-injury as a consequence of cold exposure, from those that experienced a reversible chill-coma. Behavioral and survival analysis were useful to determine the physiological state of the flies, and to corroborate the presence of chilling-injury. Moreover, we demonstrated that expression levels of stress-related genes were differentially regulated during and after the cold exposure, and that sufficient *sod* expression levels seem necessary to allow repair of cold-induced damage.

Regarding geographic distribution in temperate regions, there are contradictory reports on the overwintering potential of the medfly and its ability to survive very cold temperatures (Papadopoulos et al., 1996; Israely et al., 2004). In South America, the presence of *C. capitata* was reported as far as 40°S latitude in Argentinean Patagonia, mainly in urban areas, where they can find warm refuges during the cold winter (Oroño et al., 2005; Ovruski and Schliserman, 2012). In our study, female subgroups differed in their life expectancy, a trait that has been associated with the capacity of restoring a population after a cold period in *D. melanogaster* (Boulétreau-merle and Fouillet, 2002). After a cold season, females with a faster recovery from chill-coma and a longer life expectancy may be capable of becoming founders during favorable climate conditions. In this respect, CCR time might be considered as a physiological indicator of adaptation to cold climate in wild populations.

Acknowledgements

We would like to thank Eduardo Cafferata and Santiago Werbajh for helping with the semiquantitative RT-PCR assays. We thank Raul Alzogaray for his valuable comments and suggestions. We specially thank anonymous reviewers whose generous comments and very helpful indications allowed us to significantly improve our manuscript. This study was funded by CONICET and the University of Buenos Aires, Argentina. LMP-L was a Research Fellow of CONICET, and is currently a Postdoctoral Fellow of the Alexander von Humboldt Foundation. LAQ-A is a Full Professor at the Biological Chemistry Department, FCEyN, University of Buenos Aires. AR and LAQ-A belong to the Scientist Career of CONICET.

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