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# Chill-coma recovery time, age and sex determine lipid profiles in *Ceratitis capitata* tissues

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ABSTRACT

The remodeling of membrane composition by changes in phospholipid head groups and fatty acids (FA) degree of unsaturation has been associated with the maintenance of membrane homeostasis under stress conditions. Overall lipid levels and the composition of cuticle lipids also influence insect stress resistance and tissue protection. In a previous study, we demonstrated differences in survival, behavior and Cu/Zn superoxide dismutase gene expression between subgroups of *Ceratitis capitata* flies that had a reversible recovery from chill-coma and those that developed chilling-injury. Here, we analyzed lipid profiles from comparable subgroups of 15 and 30-day-old flies separated according to their recovery time after a chill-coma treatment. Neutral and polar lipid classes of chill-coma subgroups were separated by thin layer chromatography and quantified by densitometry. FA composition of polar lipids of chill-coma subgroups and non-stressed flies was evaluated using gas chromatography coupled to mass spectrometry. Higher amounts of neutral lipids such as triglycerides, diacylglycerol, wax esters, sterol esters and free esters were found in male flies that recovered faster from chill-coma compared to slower flies. A multivariate analysis revealed changes in patterns of storage and cuticle lipids among subgroups both in males and females. FA unsaturation increased after cold exposure, and was higher in thorax of slower subgroups compared to faster subgroups. The changes in neutral lipid patterns and FA composition depended on recovery time, sex, age and body-part, and were not specifically associated with the development of chilling-injury. An analysis of phospholipid classes showed that the phosphatidylcholine to lysophosphatidylcholine ratio (PC/LPC) was significantly higher, or showed a tendency, in subgroups that may have developed chilling-injury compared to those with a reversible recovery from coma.



## 1. Introduction

Changes in environmental temperature influence the survival, population dynamics and distribution of insects (Chown and Terblanche, 2006). When behavioral responses are not enough to cope with environmental changes, insects may adapt through physiological responses to survive (Chevin et al., 2010; Chidawanyika et al., 2012; Helmuth et al., 2005). Parker et al. (2015) recently suggested that long-term cold acclimation and short-term cold shock response may involve different physiological processes. Moreover, the response to rapid cold hardening in *Drosophila melanogaster* has been shown to protect against future unexpected temperature changes (Czajka and Lee, 1990; Shreve et al., 2004). Several physiological traits, such as thermal tolerance and resistance, have been used to study the thermal biology of insects. In particular, very little is known about the mechanisms related to chill tolerance, especially to the entrance into and recovery from comatose state (Andersen et al., 2015). The ability of an organism to react to an environmental input is called phenotypic

*Abbreviations:* CCR, chill-coma recovery; FA, fatty acids; FS, free sterols; FSG, Fast-Subgroup; ISG, Intermediate-Subgroup; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PC1, first principal component; PC2, second principal component; PCA, principal components analysis; PE, phosphatidylethanolamine; SSG, Slow-Subgroup; TLC, thin layer chromatography.

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plasticity (West-Eberhard, 2003), and can be induced within minutes or hours after a thermal shock treatment (Bowler and Terblanche, 2008). A trait used to study phenotypic plasticity is chill-coma recovery (CCR), which measures the ability of individuals to become active after being knocked down by a chilling stress (David et al., 1998; Macdonald et al., 2004). When insects enter chill-coma, there is a cessation of neuromuscular activity attributed to a disturbance of ion homeostasis (Hosler et al., 2000; Koštál et al., 2004, 2006). Chill-coma has been defined as a reversible arrest of movement (MacMillan and Sinclair, 2011) and must be distinguished from chilling-injury, where permanent damage occurs (Findsen et al., 2014). Chilling-injury is characterized by ion leakage across cell membranes and damage to intracellular organelles (Shreve et al., 2007), which can be caused by phase transition in the lipid bilayer, and a subsequent loss in membrane integrity (Lee, 1989).

Among-individual variation within a population has been suggested to be responsible for the different ability of individuals to resist stress conditions (Vaupel et al., 1979). However, the intrinsic biological variation among individuals in the same life-stage and their respective life-history have often been overlooked in thermal biology studies (Bowler and Terblanche, 2008). This variation is present even in genetically homogeneous strains of insects such as laboratory populations (Khazaeli et al., 1995), and is mainly originated by random developmental and environmental variation (Wu et al., 2006). To better understand among-individual variation associated with chill tolerance, we previously separated *Ceratitis capitata* laboratory populations in subgroups according to their CCR time, discriminating between flies that had a reversible or irreversible recovery after chill-coma (Pujol-Lereis et al., 2014). The Mediterranean fruit fly *C. capitata*, commonly referred to as medfly, has a broad thermal tolerance (Basson et al., 2012; Nyamukondiwa et al., 2010; Nyamukondiwa and Terblanche, 2009; Weldon et al., 2011) and inhabits a wide range of thermal environments throughout the tropical and sub-tropical parts of the world (Szyniszewska and Tatem, 2014). The ability of medflies to survive low temperatures is not clearly understood, with some studies showing that medflies are not able to overwinter in cold areas in Israel mountains (Israely et al., 2004), and others demonstrating overwinter potential in Greece (Papadopoulos et al., 1996). In the Southern Hemisphere, medflies were captured as far as 40°S latitude in urban areas of Argentinean Patagonia (Oroño et al., 2005; Ovruski and Schliserman, 2012), where they may have survived in warm refuges.

In the present work, we investigated whether the lipid composition of medfly CCR subgroups can reflect among-individual variation in cold susceptibility. Under cold stress, a frequent physiological response to maintain membrane fluidity is the increase in the proportion of unsaturated fatty acids (FA), and of phosphatidylethanolamine (PE) relative to phosphatidylcholine (PC) (Hazel, 1995). In *Drosophila*, changes in the PC/PE ratio and in the levels of free FA were observed as a consequence of acclimation (Koštál et al., 2011; Overgaard et al., 2007, 2008), whereas higher proportion of unsaturated FA occurred after a cold exposure (Overgaard et al., 2005), or in cold adapted flies (Ohtsu et al., 1993). Other studies showed that overall lipid contents and cuticle lipid composition are associated with stress resistance in insects (Colinet et al., 2006; Gibbs, 2002; Terblanche et al., 2008). Therefore, we hypothesized that flies with a slower recovery from chill-coma, and especially those developing chilling-injury, would have lower levels of storage and cuticle lipids (e.g. triglycerides, hydrocarbons, wax esters, and sterol esters) and/or a non-satisfactory rearrangement of phospholipid ratios or unsaturation levels comparing with individuals that recover faster. In this sense, we would expect that flies suffering from chilling-injury are not able to increase the unsaturation of FA to maintain membrane fluidity.

In *D. melanogaster*, profiles enriched in glycerophospholipids were observed in old flies (Hoffman et al., 2014), and an age-dependent elevation of the unsaturation vs. saturation index in long-lived flies was found when comparing cell membrane phospholipids FA profiles (Moghadam et al., 2013). Moreover, we showed differences in polar and neutral lipid profiles depending on age and sex in medflies (Pujol-Lereis et al., 2012). Against this background, and given that thermal resistance changes in an age-dependent manner (Bowler and Terblanche, 2008), we evaluated young (15 days old) and old (30 days old) CCR subgroups. Since chill-coma affects neural and muscle coordination (MacMillan and Sinclair, 2011), we expected to eventually detect differential changes in the lipid composition of brain, thorax muscles, and abdominal organs. In *C. capitata*, we previously observed that the effects of a mild increase in rearing temperature on lipid profiles depended on body-part (Pujol-Lereis et al., 2012). We also showed that CCR subgroups suffering chilling-injury had lower gene expression levels of Cu/Zn superoxide dismutase in thorax, but not head (Pujol-Lereis et al., 2014). It may be possible that lipid profiles also show greater alterations in thorax of injured flies. Regarding sexual dimorphism, Scheitz et al. (2013) showed for *D. melanogaster* that females have higher amounts of triglycerides, which is consistent with having additional fat body tissue than males. The researchers also showed that males have higher levels of unsaturated FA than females, but females presented a lower PC/PE ratio. These two variables have opposite effects on membrane fluidity, and their regulation under low temperatures may differ between sexes. Other study found that *Drosophila* males have higher saturated and lower polyunsaturated levels of cholesterol esters and lysophosphatidylcholine than females (Parisi et al., 2011). Therefore, we analyzed body-parts and sexes separately.

## 2. Methods

### 2.1. Fly rearing and experimental populations

We used the *C. capitata* wild-type strain Mendoza, which was originally founded with specimens taken from different host-fruits and localities of Mendoza, Argentina, and received recurrent introductions throughout successive generations (Basso et al., 2009). The climate in Mendoza is temperate warm, with an average daily temperature of 25 °C in summer and 8 °C in winter. Our original laboratory Mendoza population was a sample of around 28,000 flies, maintained in our laboratory for about 270 generations at 23 °C as previously described (Pujol-Lereis et al., 2012). For the experiments, virgin adult flies were collected less than 12 h after emergence from the puparium, sexed under mild $CO_2$ 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 five days.

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

Flies from each single-sex experimental population (see Section 2.1) that survived until the age of 15 or 30 days were subjected to CCR assay as previously described (Pujol-Lereis et al., 2014). Briefly, flies were collected from flasks under light $CO_2$ anesthesia and placed into 10 cm Petri dishes. After 30 min all flies were recovered from anesthesia, and Petri dishes were transferred to an ice container at 0 °C. After 4 h of chilling treatment, flies were

moved back to 23 °C, and immediately placed in supine position at a distance of ca. 2 cm from each other. Recovery time was recorded for each fly when it was able to stand again on its legs. Recovered flies were shortly collected in Eppendorf tubes, and were not able to assist in the recovery of other flies. Populations of 15 and 30 days of age were separated in three CCR 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 (most of them one at a time) were grouped in the FSG. After a while, sequential groups of three or more flies started recovering all together, and were grouped in the ISG, which corresponded to the mode of the population. After most of the flies had recovered, there were flies with a longer CCR time, which also recovered one at a time, and so were grouped in the SSG. Although unusual, flies that never recovered from chill-coma were discarded.

### 2.3. Extraction, separation and detection of lipid classes

Lipids from dissected heads, thoraxes (without legs and wings) and abdomens were analyzed as previously described (Pujol-Lereis et al., 2012). All the flies that recovered from chill-coma in each subgroup were used. Briefly, lipids were extracted by homogenizing in acetone, re-extracted in chloroform:methanol (3:2), and washed using a modified Folch's method (Quesada-Allué and Belocopitow, 1978). For TLC separation, silica gel 60 plates (Merck, Darmstadt, Germany) were prewashed with chloroform and activated prior to use (180 °C overnight). Neutral lipids were separated using a one-dimensional, sequential three-solvent system as follows: (i) hexane 17.5 cm from the origin (f.o.); (ii) toluene 17.5 cm f.o.; (iii) hexane:diethyl ether:acetic acid (70:30:1) 12 cm f.o. Neutral lipid classes were visualized by charring with a ferric chloride/sulfuric acid solution. To separate polar lipids, plates were developed with a one-dimensional, two-solvent system as follows: (i) chloroform:methanol:acetic acid:water (40:10:10:1) 16 cm f.o.; (ii) chloroform:methanol:acetic acid:water (120:46:19:3) 15 cm f.o. Polar lipids were detected by spraying with 5% ethanolic phosphomolibdic acid and heating for 10 min at 180 °C. Plates were exposed to ammonia vapors to whiten the background. Neutral and polar lipid standards were separated together with the samples (1,2-dipalmitoyl-sn-glycerol from Echelon Biosciences Inc., Salt Lake City, UT, USA; oleic acid, stearyl arachidate, cholesteryl palmitate, L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, L-α-lysophosphatidylcholine, glyceryl trioleate, and cholesterol from Sigma-Aldrich Co., St. Louis, MO, USA). All solvents used were HPLC grade. Typical chromatograms are shown in Supplementary Fig. S1, and relative retardation factors of the lipid classes in Supplementary Table S1. Densitometry of lipid classes was performed using ImageJ/Fiji software (fiji.sc), and the area under the peak for each spot was used to quantify in terms of arbitrary units. Three independent replicates were analyzed for each group.

### 2.4. Polar lipid fatty acid composition analyses

Lipid extracts from SSG, FSG, and non-stressed flies were separated in silica gel 60 aluminum sheets (Merck, Darmstadt, Germany) using hexane:diethyl ether:acetic acid (70:30:1) as solvent system. The silica from the origin spots corresponding to polar lipids was scraped off and lipids were re-extracted by two sequential elutions with chloroform:methanol:water (5:5:1) followed by a centrifugation step (9000 rpm, 30 min) (Rodríguez, 2008). The supernatants were pooled, dried under nitrogen flow and stored at -20 °C until use. Polar lipid extracts were transmethylated with 300 μL of 1.3 M boron trifluoride in methanol (Fluka, Sigma-Aldrich Co., USA) at 65 °C during 180 min. After incubation, 1 mL of distilled water was added and the fatty acid methyl esters were extracted with 1 mL of hexane. This phase was injected onto a Perkin Elmer Clarus 560D MS–gas chromatograph equipped with a mass selective detector with quadrupole analyzer and photomultiplier detector and a split/splitless injector. In the gas chromatographic system, an Elite 5MS (Perkin Elmer) capillary column (30 m, 0.25 mm ID, 0.25 μm df) was used. Column temperature was programmed from 130 to 250 °C at a rate of 5 °C/min and 250 °C for 6 min. Injector temperature was set to 260 °C and inlet temperature was kept at 250 °C. Split injections were performed with a 10:1 split ratio. Helium carrier gas was used at a constant flow rate of 1 mL/min. In the mass spectrometer, electron ionization (EI+) mass spectra were recorded at 70 eV ionization energy in full scan mode (50-400 unit mass range). The ionization source temperature was set at 180 °C. The FA composition of the lipid extracts was determined by comparing their methyl derivatives mass fragmentation patterns with those of mass spectra from the NIST databases. Three independent replicates were analyzed for each group.

### 2.5. Statistical analyses

Principal component analyses (PCAs), analyses of variance (ANOVAs) and Student *t*-tests were done using the Infostat 2010 Software (UNC, Córdoba, Argentina). Blocked ANOVAs were carried out when necessary. PCAs were carried out using the densitometric values of the lipid classes. PCAs were based on correlation matrices, and the minimum eigenvalue was set at 1. Separate analyses were done for each sex and body-part. The *loadings* of the principal components (Table 1) are the correlations of each variable on each principal component. Therefore, lipid classes with higher *loadings* (either positive or negative) are more closely related to the patterns revealed by the principal components.

## 3. Results

### 3.1. Subgroups with reversible and irreversible damage

*C. capitata* single-sex experimental populations maintained at 23 °C were subjected to a CCR assay at 15 and 30 days of age, and separated into three CCR subgroups: Fast-Subgroup (FSG), Intermediate-Subgroup (ISG), and Slow-Subgroup (SSG) (see Section 2.2). It was considered that subgroups of flies within a population that had a slow and highly variable recovery after chill-coma had suffered chilling-injury (MacMillan et al., 2012; Pujol-Lereis et al., 2014). SSG in 15 and 30-day-old females, and 30-day-old males (Fig. 1A, B and D; black arrows) may have suffered irreversible chilling-injury. The other subgroups seemed to have experienced a reversible damage, including all 15-day-old male subgroups (Fig. 1C). These results are in accordance with our previous studies (Pujol-Lereis et al., 2014).

### 3.2. Lipid profiles of the CCR subgroups

Lipid classes were measured to investigate possible differences among CCR subgroups. The analyses were performed in heads (mainly brain tissue), thoraxes (mainly muscle) and abdomens. Several neutral lipid classes showed age-dependent changes in both sexes (Figs. 2–4). In addition, significant differences were found among CCR male subgroups, and interesting tendencies were also observed in females (Figs. 3 and 4).

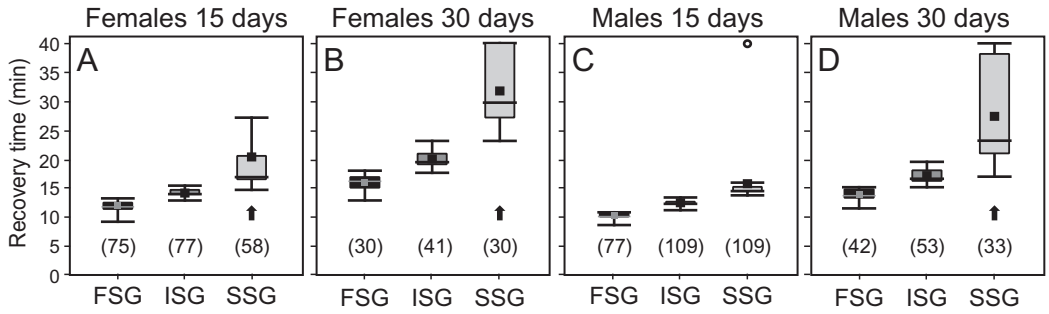


**Fig. 1.** Chill-coma recovery time of *C. capitata* subgroups. Boxplots of the recovery time (min) of female and male subgroups at 15 and 30 days of age. The upper and lower ends of the center box indicate the 75th and 25th percentiles, respectively. The line inside the box indicates the median and the bars indicate 1.5 times the distance of the interquartile range from the median. Black square dots mark the mean, and white circles mark potential outliers (more than 3 S.D. from the mean). Values from three independent populations were pooled per sex and age. Numbers in brackets indicate the total number of individuals. Black arrows show subgroups that may have suffered chilling-injury. FSG, Fast-Subgroup (black); ISG, Intermediate-Subgroup (dark gray); SSG, Slow-Subgroup (light gray).

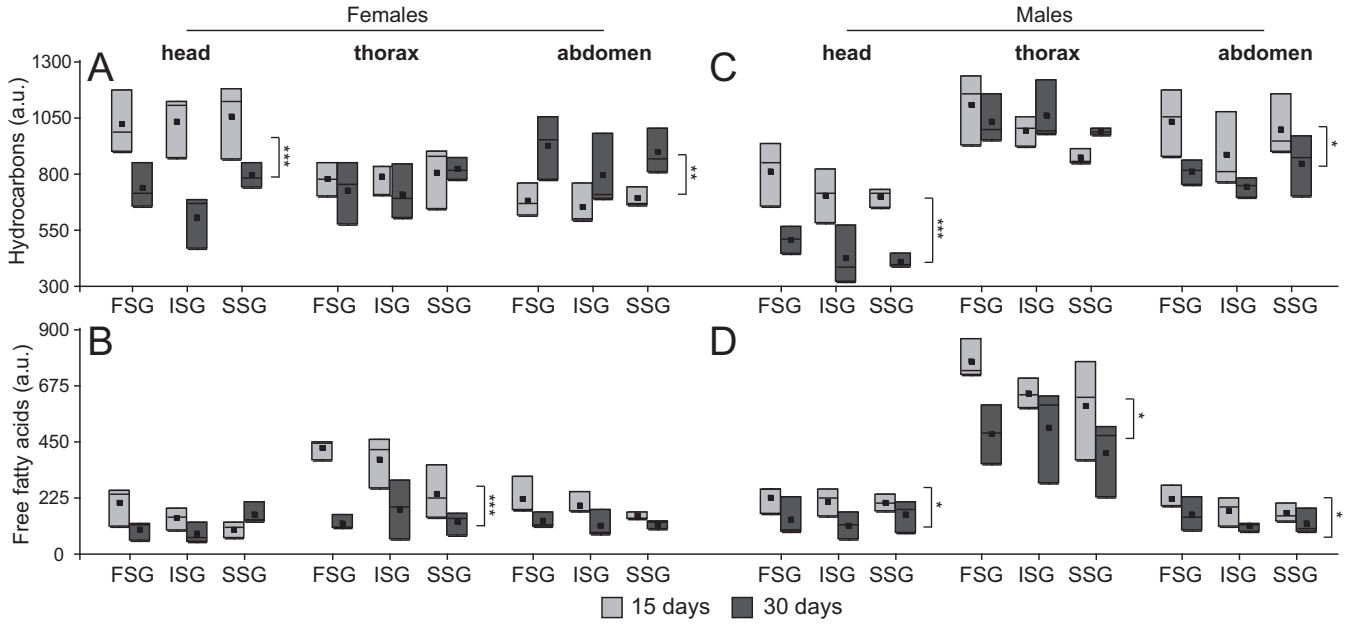


**Fig. 2.** Hydrocarbons and free fatty acids of medfly subgroups. Lipid levels (a.u., densitometric arbitrary units) from head, thorax and abdomen of 15 (light gray) and 30-day-old (dark gray) females (A and B) and males (C and D). FSG, Fast-Subgroup; ISG, Intermediate-Subgroup; SSG, Slow-Subgroup. Each value corresponds to the mean of three replicates (±S.E.M.). Statistics: two-way ANOVAs followed by Tukey's test; asterisks indicate significant differences between ages ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). No interactions were found in these analyses. See boxplots explanation in Fig. 1.

Hydrocarbons and free FA showed significant changes only with age (Fig. 2). Lower levels of hydrocarbons were seen in 30-day-old compared to 15-day-old flies in heads (both sexes, Fig. 2A and C) and abdomens (males, Fig. 2C). In female abdomens the trend is opposite, with higher levels of hydrocarbons in older flies (Fig. 2A). Free FA levels decreased with age in thoraxes of females (Fig. 2B) and in all body-parts of males (Fig. 2D).

Levels of wax esters, sterol esters and free sterols in the CCR subgroups are shown in Fig. 3. Age-dependent changes were seen in wax esters in female heads (Fig. 3A), sterol esters in male thoraxes and abdomens (Fig. 3E), and free sterols in female thoraxes (Fig. 3C). A decrease in free sterols was also observed for male thoraxes only in FSG (Fig. 3F). In addition, differences among the CCR subgroups were found. Sterol esters in male FSG presented higher levels than in SSG (Fig. 3E), with the same tendency in female thoraxes (ANOVA $p = 0.07$; Fig. 3B). Free sterols were significantly lower in SSG compared to FSG only in thoraxes of 15-day-old males (Fig. 3F). Lower levels of lipids in male SSG compared to FSG were also seen for wax esters in heads (Fig. 3D). No significant differences were found among subgroups in waxes and sterols of females (Fig. 3A–C).

Fig. 4 shows the results for triglycerides and diglycerides. We found age-dependent changes in all body-parts of both sexes for triglycerides, which is the most abundant lipid class in the medfly (Fig. 4A and D). We also observed lower levels of diglycerides in heads of older flies for both sexes (Fig. 4B, C, E and F), and of 1,2-diacylglycerol in thoraxes and abdomens of females (Fig. 4C). No significant differences were found among female subgroups for these lipid classes (Fig. 4A–C). Males presented lower levels of triglycerides in SSG compared to FSG in all body-parts (Fig. 4D). The same was observed for 1,3-diacylglycerol in heads and thoraxes (Fig. 4E), and 1,2-diacylglycerol in heads (Fig. 4F).

To better understand the overall relationship among neutral lipids related to membrane and storage homeostasis, we carried out a multivariate analysis and found two principal components (PC1 and PC2) for each body-part and sex showing interesting tendencies (Fig. 5). The *loadings* in Table 1 show that most of the neutral lipid classes had a high positive correlation with PC1 in both

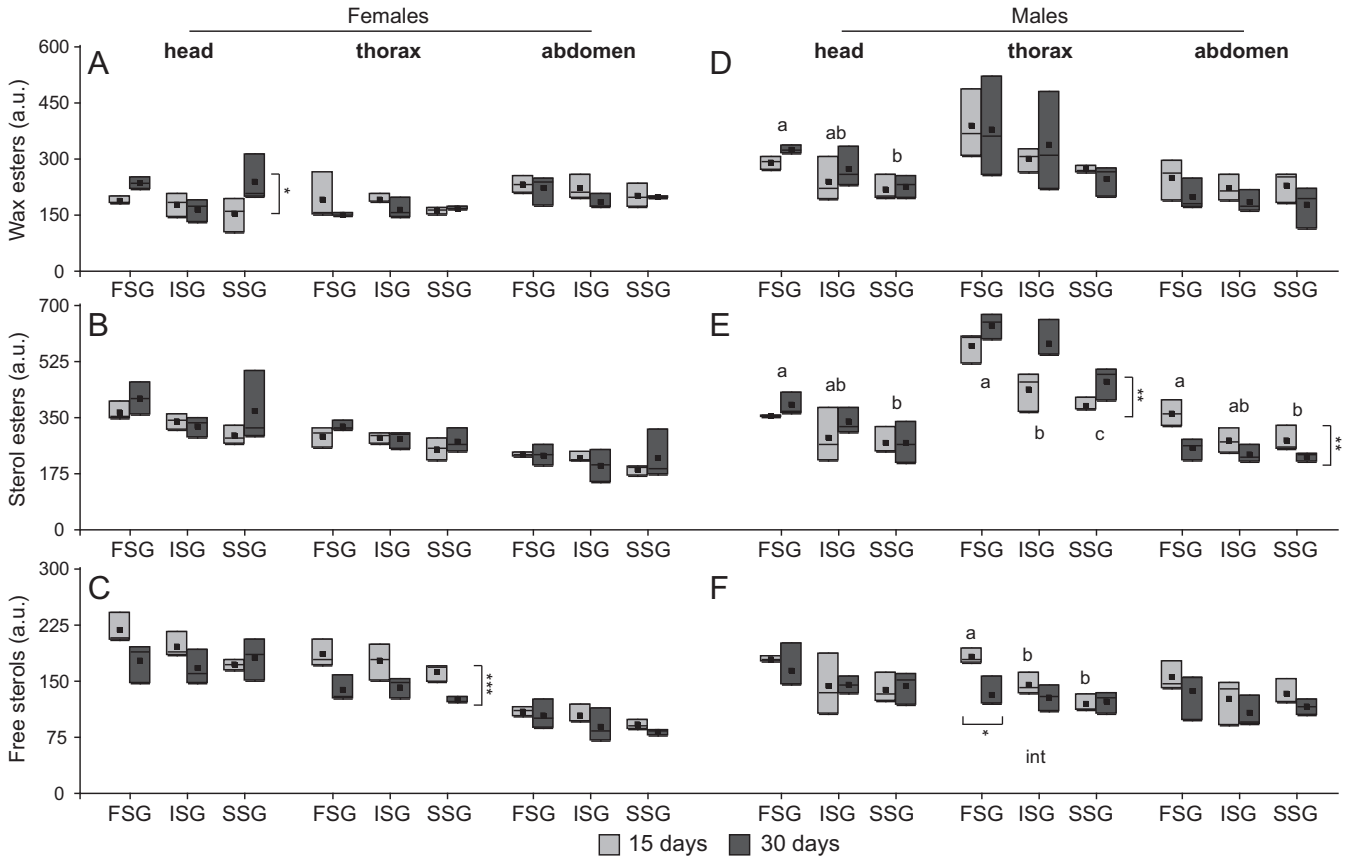


**Fig. 3.** Waxes and sterols composition of medfly subgroups. Lipid levels (a.u., densitometric arbitrary units) from head, thorax and abdomen of 15 (light gray) and 30-day-old (dark gray) females (A–C) and males (D–F). FSG, Fast-Subgroup; ISG, Intermediate-Subgroup; SSG, Slow-Subgroup. Each value corresponds to the mean of three replicates (±S.E.M.). Statistics: two-way ANOVAs followed by Tukey's test; different letters indicate significant differences among subgroups ($p < 0.05$); asterisks indicate significant differences between ages ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); when significant interaction (int), simple effect tests were carried out. See boxplots explanation in Fig. 1.

sexes, representing broad changes in neutral lipid contents. In females, PC1 was significantly different between 15 and 30-day-old flies, with older flies having fewer amounts of lipids (Fig. 5A). In males, PC1 also reflected age-dependent changes in heads and abdomens, and, more interestingly, changes among CCR subgroups in heads and thoraxes (Fig. 5C). PC2 presented higher correlation with specific lipid classes depending on sex and body-part, but sterol esters and wax esters dominated these patterns (Table 1). In female thoraxes, PC2 differed significantly among subgroups (Fig. 5B), showing a pattern of higher sterol esters and lower hydrocarbons in FSG compared to SSG. In male heads (Fig. 5D), higher sterol esters and wax esters levels were detected in FSG compared to SSG as well as in young compared to old flies. Biplot diagrams of PC1 vs. PC2 show the discrimination between SSG and FSG according to the lipid classes (Fig. 5E).

Ratios of phosphatidylcholine (PC) to phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC) and free sterols (FS) were analyzed between FSG and SSG (Table 2). We found a higher PC/FS ratio in SSG compared to FSG in heads and thoraxes of young males, and abdomens of old females. PC/LPC ratio was also higher in SSG compared to FSG in heads of 30-day-old female flies, and the same tendency was observed for old males and young females.

### 3.3. Polar lipid fatty acid composition of the CCR subgroups

We next evaluated if, as a consequence of the chill-coma, subgroups also presented different FA composition. We compared polar lipid FA of FSG, SSG, and flies non-exposed to cold.

In 30-day-old females subjected to CCR assay, heads presented a decrease of 18:1 n9 and an increase of 18:2 n6 compared to non-stressed flies, but no differences were observed between CCR subgroups (Table 3). Thoraxes presented a decrease of 16:1 n9 and an increase of 18:2 n6, with control levels significantly different from SSG, and FSG with intermediate levels. Similar tendencies were seen in 15-day-old females (Table 3).

Males also showed significant differences among controls and CCR subgroups, and between CCR subgroups. In male heads there was a decrease of 18:1 n9 (15 and 30 days) and an increase of 18:2 n6 (30 days) in the groups subjected to cold compared to controls (Table 3). In thoraxes of 15-day-old males, FSG and SSG showed a lower proportion of 16:1 n9, and a higher proportion of 18:2 n6 compared to controls, being these changes more pronounced in SSG; while 16:0 was significantly lower in SSG comparing with FSG and controls (Table 3). In thoraxes of 30-day-old males, FSG and SSG showed a higher proportion of 18:2 n6 compared to the control, and again changes were more pronounced in SSG. SSG had a lower proportion of 18:1 n9 regarding to FSG and the control, and 16:1 n9 in SSG was significantly lower compared to the control, whereas FSG had intermediate levels (Table 3).

In summary, we found an increase in FA unsaturation in heads and thoraxes after cold exposure, which may enable the maintenance of membrane fluidity. Moreover, these changes were higher in thoraxes of SSG.

## 4. Discussion

We previously demonstrated that subgroups of flies within a population that had a slow and highly variable recovery after chill-coma developed chilling-injury, while the others experienced

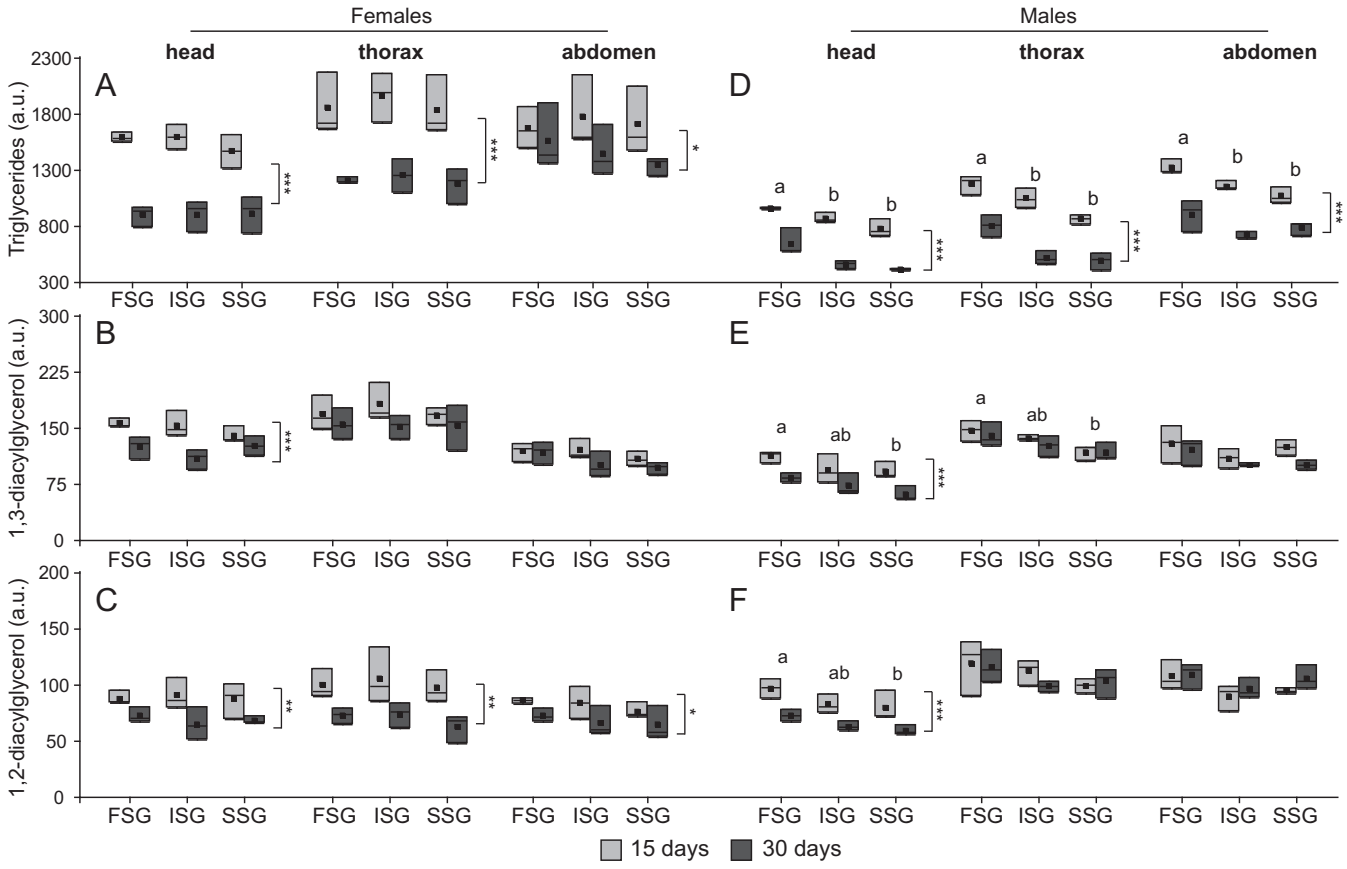


**Fig. 4.** Tri- and diglycerides of medfly subgroups. Lipid levels (a.u., densitometric arbitrary units) from head, thorax and abdomen of 15 (light gray) and 30-day-old (dark gray) females (A–C) and males (D–F). FSG, Fast-Subgroup; ISG, Intermediate-Subgroup; SSG, Slow-Subgroup. Each value corresponds to the mean of three replicates (±S.E.M.). Statistics: two-way ANOVAs followed by Tukey's test; different letters indicate significant differences among subgroups ($p < 0.05$); asterisks indicate significant differences between ages ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). No interactions were found in these analyses. See boxplots explanation in Fig. 1.

a reversible recovery (Pujol-Lereis et al., 2014). In order to further investigate the physiological changes of *C. capitata* after a rapid cold exposure, we used the same approach to separate 15 and 30-day-old experimental populations in CCR subgroups, and analyze their lipid profiles.

When analyzing single lipid classes, several neutral lipid classes showed age-dependent changes in both sexes, in agreement with our previous results (Pujol-Lereis et al., 2012). Regarding CCR subgroups, we observed a higher amount of lipids in male FSG compared to SSG for both ages, i.e. triglycerides and sterol esters in all body-parts, wax esters and 1,2-diacylglycerol in heads, and 1,3-diacylglycerol in heads and thoraxes. Diacylglycerol is a second messenger that regulates synaptic activity, and the *Drosophila* diacylglycerol kinase that converts diacylglycerol to phosphatidic acid is highly expressed in the central nervous system and indirect flight muscles (Masai et al., 1992). In plants, diacylglycerol kinase regulates the levels of these lipid messengers under cold temperatures (Arisz et al., 2013). The fact that 1,2-diacylglycerol presented lower levels in heads of male SSG compared to FSG, and only a slight tendency in thorax, may reflect a differential response to

**Table 1**
PCA *loadings* representing the correlation of lipid classes with PC1 and PC2.

| Lipid class | PC1 | | | | | | PC2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Females | | | Males | | | Females | | | Males | | |
| | Head | Tho | Abd | Head | Tho | Abd | Head | Tho | Abd | Head | Tho | Abd |
| Hydrocarbons | **0.79** | 0.48 | 0.07 | **0.82** | **0.80** | **0.80** | −0.15 | **−0.66** | **0.87** | −0.48 | −0.44 | −0.42 |
| Sterol esters | −0.06 | 0.07 | 0.48 | 0.55 | 0.58 | **0.84** | **0.94** | **0.68** | **0.66** | **0.81** | **−0.60** | 0.01 |
| Wax esters | −0.15 | **0.71** | **0.83** | 0.51 | **0.84** | **0.65** | **0.86** | −0.01 | 0.00 | **0.81** | −0.36 | **−0.64** |
| Triglycerides | **0.89** | **0.93** | **0.84** | **0.86** | **0.62** | **0.85** | −0.31 | 0.01 | −0.17 | −0.40 | **0.64** | −0.23 |
| Free fatty acids | 0.58 | **0.76** | **0.88** | **0.76** | 0.58 | **0.88** | 0.53 | 0.36 | −0.18 | −0.28 | **0.66** | 0.29 |
| Free sterols | **0.74** | **0.95** | **0.91** | **0.76** | **0.82** | **0.90** | 0.51 | 0.20 | 0.23 | 0.53 | 0.31 | 0.37 |
| 1,3-Diacylglycerol | **0.93** | **0.85** | **0.86** | **0.96** | **0.95** | **0.89** | −0.05 | −0.26 | 0.04 | −0.11 | 0.02 | −0.01 |
| 1,2-Diacylglycerol | **0.86** | **0.94** | **0.83** | **0.95** | **0.75** | 0.49 | −0.06 | 0.03 | −0.38 | −0.22 | −0.06 | **0.74** |
| Variability (%)[a] | 49 | 58 | 58 | 62 | 57 | 64 | 29 | 14 | 18 | 26 | 20 | 18 |

Abbreviations: abd, abdomen; thor, thorax; PC1, first principal component; PC2, second principal component.
Numbers in bold: absolute values higher than 0.60.
[a] Amount of variability in the original variables explained by the principal component.

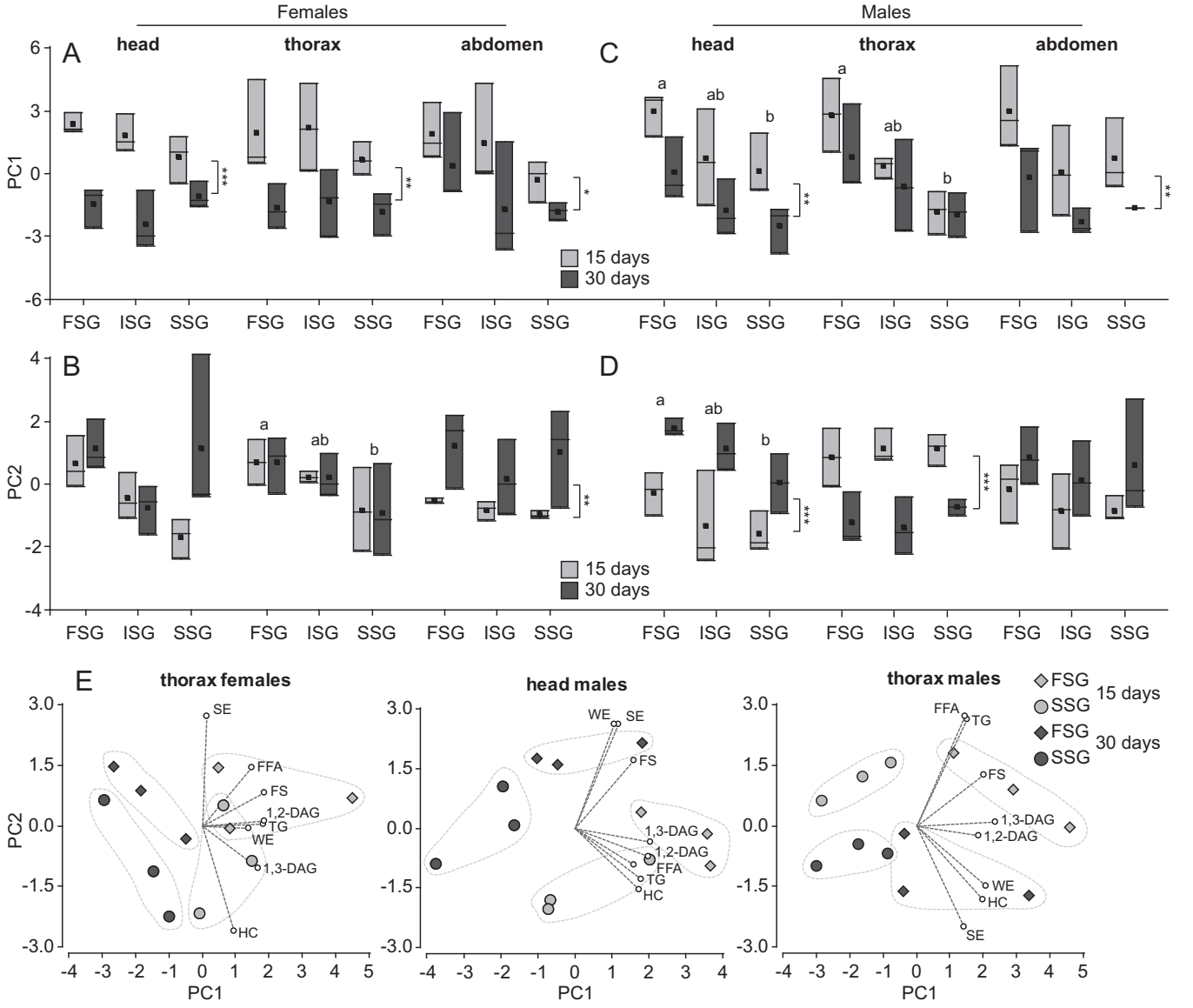


**Fig. 5.** Principal component analysis (PCA) of neutral lipids. (A) PC1 of females, (B) PC2 of females, (C) PC1 of males, (D) PC2 of males, (E) biplots of PC1 vs. PC2. PCAs were run for each sex and body-part using the densitometric values of neutral lipid classes of 15 (light gray) and 30-day-old (dark gray) fly subgroups. FSG, Fast-Subgroup; ISG, Intermediate-Subgroup; SSG, Slow-Subgroup. (A–D) Each value corresponds to the mean of three replicates (±S.E.M.). Statistics: two-way ANOVAs followed by Tukey's test; different letters indicate significant differences among subgroups ($p < 0.05$); asterisks indicate significant differences between ages ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). No interactions were found in these analyses. See boxplots explanation in Fig. 1. (E) Biplot diagrams of PC1 vs. PC2 are shown for those analyses that gave statistical differences among subgroups. Samples of FSG and SSG are displayed as rhombus and circles, respectively, while variables are displayed as vectors (dashed lines with small circles). Samples corresponding to the same subgroup are surrounded by a dashed gray line. The length of a vector is proportional to the variance of the corresponding variable. The angle between the vectors approximates the correlation between the variables they represent. Abbreviations: DAG, diacylglycerol; FFA, free fatty acids; FS, free sterols; HC, hydrocarbons; SE, sterol esters; TG, triglycerides; WE, wax esters.

cold among the subgroups at a nervous system level. Thorax free sterols were the only lipid class with higher levels in FSG compared to SSG in young males, but not in old flies, which may reflect a higher among-individual variability for free sterols in young adult life-stages. When applying a multivariate approach, PC1 of male heads and thoraxes revealed differences among the CCR subgroups in total amount of lipids. Studies with the wasp *Aphidius colemani* showed that energy reserves are crucial for survival in extreme temperatures (Colinet et al., 2006). Consistent with these results, Terblanche et al. (2008) found that *Glossina pallidipes* flies that are more resistant to cold also had higher contents of lipids and water. Therefore, the high correlation of male PC1 with triglycerides and other lipid classes may reflect the energy capacity and ability to resist low temperatures of the flies, which negatively correlates with their recovery time. The fact that no significant differences in neutral lipid classes were observed among female CCR subgroups may be associated with a fine-tune regulation of energy storages necessary for specific reproductive processes.

Another interesting pattern was unraveled by male head PC2, which presented high positive correlations with sterol esters and wax esters, and negative correlations with hydrocarbons and triglycerides. This component was higher in FSG compared to SSG. In females, the only principal component that was significantly higher in FSG compared to SSG was thorax PC2, which was also positively correlated with sterol esters and negatively correlated with hydrocarbons. Insect's cuticle is mainly composed of hydrocarbons, and also contains other lipids such as wax esters, sterol esters and triglycerides (Gołębiowski et al.,

**Table 2**
Lipid classes' ratios (mean ± S.E.M.). Comparison of Fast-Subgroup (FSG) and Slow-Subgroup (SSG).

| | | Head | | Thorax | | Abdomen | |
|---|---|---|---|---|---|---|---|
| | | FSG | SSG | FSG | SSG | FSG | SSG |
| Females 15 days | PC/PE | 0.53 ± 0.03 | 0.56 ± 0.04 | 0.76 ± 0.07 | 0.66 ± 0.06 | 0.90 ± 0.10 | 0.90 ± 0.04 |
| | PC/LPC | 5.96 ± 0.94 | 7.40 ± 0.80 | 7.45 ± 0.80 | 13.21 ± 3.68 | 5.71 ± 0.69 | 7.12 ± 0.43 |
| | PC/FS | 3.13 ± 0.41 | 4.19 ± 0.37 | 20.25 ± 2.54 | 22.94 ± 0.92 | 9.86 ± 0.46 | 11.92 ± 0.98 |
| Females 30 days | PC/PE | 0.47 ± 0.02 | 0.44 ± 0.02 | 0.52 ± 0.03 | 0.49 ± 0.01 | 0.88 ± 0.08 | 0.83 ± 0.11 |
| | PC/LPC | **13.65 ± 0.83** | **24.33 ± 3.24*** | 26.87 ± 7.62 | 27.04 ± 8.29 | 13.13 ± 3.17 | 14.68 ± 3.59 |
| | PC/FS | 2.88 ± 0.30 | 2.84 ± 0.36 | 23.18 ± 3.18 | 25.80 ± 0.39 | **9.11 ± 0.56** | **11.22 ± 0.21*** |
| Males 15 days | PC/PE | 0.51 ± 0.04 | 0.52 ± 0.05 | 0.57 ± 0.02 | 0.63 ± 0.02 | 0.65 ± 0.05 | 0.69 ± 0.01 |
| | PC/LPC | 7.01 ± 0.79 | 7.17 ± 0.64 | 5.55 ± 0.23 | 5.11 ± 0.44 | 4.53 ± 0.26 | 4.73 ± 0.14 |
| | PC/FS | **4.92 ± 0.24** | **7.16 ± 0.42**** | **12.09 ± 1.08** | **18.34 ± 0.66**** | 6.29 ± 0.39 | 7.22 ± 0.38 |
| Males 30 days | PC/PE | 0.42 ± 0.01 | 0.38 ± 0.02 | 0.52 ± 0.02 | 0.50 ± 0.03 | 0.53 ± 0.02 | 0.50 ± 0.01 |
| | PC/LPC | 26.74 ± 6.93 | 53.74 ± 7.98 | 8.74 ± 1.03 | 14.95 ± 2.64 | 7.94 ± 1.32 | 13.05 ± 4.43 |
| | PC/FS | 6.18 ± 0.60 | 5.61 ± 0.64 | 13.09 ± 1.03 | 13.16 ± 2.08 | 6.78 ± 1.14 | 6.02 ± 0.29 |

Abbreviations: FS, free sterols; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
Statistics: Student *t*-tests (*$p < 0.05$, **$p < 0.01$; highlighted in bold font).

**Table 3**
Fatty acid composition (% ± S.E.M.) of polar lipids. Comparison of non-stressed flies (no CCR), Fast-Subgroup (FSG) and Slow-Subgroup (SSG).

| | | Head | | | Thorax | | |
|---|---|---|---|---|---|---|---|
| | | No CCR | FSG | SSG | No CCR | FSG | SSG |
| Females 15 days | 16:0 | 15.0 ± 1.1 | 14.6 ± 1.1 | 15.5 ± 0.7 | 12.5 ± 0.7 | 10.9 ± 0.8 | 11.2 ± 0.1 |
| | 16:1 n9 | 3.7 ± 0.2 | 3.6 ± 0.6 | 2.5 ± 0.8 | 22.2 ± 1.6 | 20.4 ± 1.7 | 21.9 ± 1.5 |
| | 18:0 | 39.2 ± 1.2 | 40.5 ± 1.4 | 51.4 ± 8.6 | 6.5 ± 2.8 | 15.1 ± 4.5 | 11.0 ± 1.8 |
| | 18:1 n9 | 32.4 ± 1.6 | 27.7 ± 1.2 | 19.8 ± 6.0 | 42.6 ± 0.8 | 37.3 ± 2.5 | 38.5 ± 0.7 |
| | 18:2 n6 | 9.6 ± 0.9 | 13.6 ± 1.2 | 10.9 ± 2.4 | 16.3 ± 0.3 | 16.3 ± 1.5 | 17.4 ± 0.3 |
| Females 30 days | 16:0 | 15.4 ± 0.4 | 15.5 ± 2.3 | 12.6 ± 0.7 | 11.4 ± 0.3 | 11.4 ± 0.5 | 11.5 ± 0.9 |
| | 16:1 n9 | 3.4 ± 0.3 | 2.4 ± 0.5 | 2.1 ± 0.1 | **25.9 ± 0.1$^{a}$** | **22.1 ± 1.0$^{ab}$** | **20.7 ± 1.6$^{b}$** |
| | 18:0 | 42.3 ± 0.2 | 43.9 ± 1.3 | 46.3 ± 1.9 | 9.3 ± 0.3 | 11.9 ± 1.0 | 12.9 ± 1.2 |
| | 18:1 n9 | **31.3 ± 0.1$^{a}$** | **25.9 ± 1.1$^{b}$** | **26.4 ± 1.4$^{b}$** | 40.2 ± 0.6 | 38.4 ± 1.4 | 37.0 ± 0.9 |
| | 18:2 n6 | **7.8 ± 0.5$^{a}$** | **12.2 ± 0.6$^{b}$** | **12.6 ± 0.6$^{b}$** | **13.2 ± 0.1$^{a}$** | **16.2 ± 1.2$^{ab}$** | **18.0 ± 0.3$^{b}$** |
| Males 15 days | 16:0 | 13.4 ± 0.2 | 11.7 ± 0.1 | 13.4 ± 1.5 | **11.1 ± 0.5$^{a}$** | **10.7 ± 0.3$^{a}$** | **8.9 ± 0.3$^{b}$** |
| | 16:1 n9 | 4.1 ± 0.4 | 3.2 ± 0.7 | 2.6 ± 1.3 | **22.6 ± 0.5$^{a}$** | **19.3 ± 0.4$^{b}$** | **16.9 ± 0.6$^{c}$** |
| | 18:0 | 42.5 ± 0.3 | 50.4 ± 2.3 | 50.2 ± 5.0 | 7.9 ± 0.4 | 11.4 ± 1.2 | 11.5 ± 1.1 |
| | 18:1 n9 | **30.1 ± 0.2$^{a}$** | **23.4 ± 1.4$^{b}$** | **24.0 ± 2.4$^{b}$** | 42.6 ± 0.2 | 40.3 ± 0.7 | 41.8 ± 0.9 |
| | 18:2 n6 | 9.9 ± 0.3 | 11.4 ± 0.1 | 9.8 ± 1.7 | **15.8 ± 0.3$^{a}$** | **18.3 ± 0.4$^{b}$** | **20.9 ± 0.7$^{c}$** |
| Males 30 days | 16:0 | 12.8 ± 0.7 | 14.3 ± 0.7 | 13.2 ± 1.1 | 10.9 ± 0.4 | 10.5 ± 0.5 | 10.0 ± 0.4 |
| | 16:1 n9 | 3.8 ± 0.3 | 4.8 ± 1.2 | 3.1 ± 0.1 | **23.8 ± 1.3$^{a}$** | **20.7 ± 1.3$^{ab}$** | **18.0 ± 1.2$^{b}$** |
| | 18:0 | 41.8 ± 1.1 | 40.8 ± 1.7 | 45.2 ± 0.6 | 9.2 ± 0.9 | 11.9 ± 1.1 | 12.9 ± 1.7 |
| | 18:1 n9 | **32.2 ± 0.5$^{a}$** | **27.6 ± 1.4$^{b}$** | **27.2 ± 0.9$^{b}$** | **41.8 ± 0.7$^{a}$** | **41.5 ± 0.7$^{a}$** | **38.9 ± 0.7$^{b}$** |
| | 18:2 n6 | **9.4 ± 0.5$^{a}$** | **12.4 ± 0.8$^{b}$** | **11.4 ± 0.2$^{ab}$** | **14.4 ± 0.3$^{a}$** | **15.5 ± 0.1$^{b}$** | **20.2 ± 0.3$^{c}$** |

Statistics: one-way ANOVAs followed by Tukey's test; different letters indicate significant differences among groups ($p < 0.05$; highlighted in bold font).

2011). This lipid containing surface is responsible of the water balance of insects, and also protects against chemicals, toxins, microorganisms and predators. Moreover, wax esters of insect cuticle have been related to the prevention of tissue stress (Gibbs, 2002). Thus, the pattern revealed by PC2 may reflect among-individual variability in the cuticle composition of the flies, and in their ability to cope with environmental stressors such as temperature variations.

Changes in the composition and structure of lipids upon variation of temperature are crucial to maintain membrane fluidity (Hazel, 1995). The characteristics of phospholipid head groups influence the direction of these changes. PC has a cylindrical head group that favors close packing of lipids, while PE has a more conical conformation and counteracts the ordering of membranes at low temperatures (Hazel, 1989), and therefore the PC/PE ratio decreases as temperature falls (Barrett, 2011). In *Drosophila*, a restructuring of the glycerophospholipid composition was observed as a consequence of acclimation to high and low temperatures, with changes in the relationship between PC and PE, and also a decrease of free FA (Koštál et al., 2011; Overgaard et al., 2007, 2008). PC/PE ratio was not different between the CCR subgroups, which may indicate that fast and slow flies had the same capacity to maintain the main phospholipid composition of membranes under cold exposure. Although we haven't made a comparison of the PC/PE ratio with non-stressed flies, our findings are in agreement with results from Macmillan et al. (2009) and Koštál et al. (2003) in Drosophilidae species that found no correlation between levels of PC and PE and freeze tolerance. On the other side, PC/LPC ratio was significantly higher in the head of SSG of old females compared to FSG, with the same tendency in body-parts of young females and old males, in which SSG have suffered chilling-injury. This tendency has not been observed in young males SSG that experienced reversible chill-coma. LPC is generated by hydrolysis of PC, and has been associated with calcium mobilization in *Drosophila* cells (Wang et al., 2015). A higher PC/LPC ratio can cause a disruption of calcium homeostasis that may be involved in the irreversible chilling damage in these subgroups of flies.

An increase in the cholesterol levels in the diet improved *D. melanogaster* survival following a cold stress, which was associated with an improvement in membrane fluidity (Shreve et al., 2007). However, depending on the species and tissue evaluated, levels

of cholesterol can increase or decrease during cold acclimation (Crockett, 1998). In our study we have observed a higher PC/FS ratio in male young SSG compared to FSG. We have previously demonstrated that 15-day-old male CCR subgroups presented no differences in survival, locomotor activity or superoxide dismutase gene expression (Pujol-Lereis et al., 2014). As cholesterol stabilizes liquid crystal phase and inhibits gel phase formation (Barrett, 2011), the lower amount of free sterols in male young SSG may impact the time required for these flies to recover after a cold stress, but is not associated with the development of chilling-injury.

As a last approach to the understanding of lipid differences between CCR subgroups, we measured the FA composition of polar lipids, previously associated with cold stress and survival (Hulbert et al., 2007; Overgaard et al., 2005). Results from thoraxes showed significant differences between the CCR subgroups whereas no differences or slight tendencies were seen in heads. When individuals were subjected to CCR assay, there was a decrease in the proportion of monounsaturated FA (16:1 n9, 18:1 n9) and an increase in the proportion of polyunsaturated FA (18:2 n6). These results are in agreement with previously described changes after cold exposure in *Drosophila* flies (Overgaard et al., 2005), which served to maintain membrane fluidity. Differences in the FA composition between CCR subgroups were significant in male thoraxes at both ages, and similar trends were obtained in 15-day-old female thoraxes. Moreover, changes were higher in SSG, the subgroups that recovered slower after chill-coma. It may be possible that the stress affecting membranes is higher in the thorax of SSG, and therefore the response to maintain membrane fluidity is exacerbated. This tissue sensitivity is in agreement with our previous results showing lower expression levels of the antioxidant gene *sod* in thoraxes, but not heads, of SSG subgroups (Pujol-Lereis et al., 2014).

In summary, our study demonstrated that subgroups of flies presented differences in lipid classes according to their recovery time and age. We also showed that flies that recovered slower from chill-coma had a higher increase in polyunsaturated FA together with a decrease in monounsaturated and saturated FA. Only for the PC/LPC ratio we observed a significant difference or tendency between subgroups that may have developed chilling-injury and subgroups with a reversible recovery from chill-coma.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2016.02.002.

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