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Lipid profiles as indicators of functional senescence in the medfly

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ABSTRACT

Changes associated with the age-related decline of physiological functions, and their relation with mortality rates, are thoroughly being investigated in the aging research field. We used the Mediterranean fruit fly *Ceratitis capitata*, largely studied by biodemographers, as a model for functional senescence studies. The aim of our work was to find novel combinatorial indicators able to reflect the functional state of adult insects, regardless of chronological age. We studied the profiles of neutral and polar lipids of head, thorax and abdomen of standard populations kept at 23 °C, at different ages. Lipid classes were separated by thin layer chromatography, and the quantitative values were used to find patterns of change using a multivariate principal component analysis approach. The lipid-dependent principal components obtained correlated with age, and differences between sexes were consistent with differences in the shape of the survival curves and the mortality parameters. These same components were able to discriminate populations with a behavioral decline due to a mild 28 °C thermal stress. Thus, young populations at 28 °C showed similar lipid profiles than old populations at 23 °C. The results indicated that the lipid-dependent components reflect the functional state of the flies, and so were named functional state components (FSCs). It is proposed that FSCs may be used as functional senescence indicators.

1. Introduction

Aging is a combination of external and internal factors acting upon the genetic background of an individual within a population, thus contributing to the overall senescence parameters of that population (Burger and Promislow, 2006; Kenyon, 2010). The aging process implies a progressive deterioration of the capacity to maintain homeostasis and, consequently, the physiological functions (Toroser et al., 2007). In the last years, the study of functional senescence, the intrinsic age-related decline in functional status, was able to identify key organ systems that fail with age, some of which might be directly involved in mortality (Grotewiel et al., 2005). Transcriptome analysis of *Drosophila melanogaster* using different body parts suggested that muscles might be particularly sensitive to aging (Girardot et al., 2006). Other works in *Drosophila* correlated behaviors that undergo age-related decline, such as locomotor activity, geotaxis, circadian rhythms and cognitive functions (Simon et al., 2006; Iliadi and Boulianne, 2010) with molecular-genetic determinants. For example, it has been proposed that functional senescence and age-dependent mortality are influenced by β-integrins (Goddeeris et al., 2003), whereas changes in dopamine levels have been associated with longevity and locomotor activity (Vermeulen et al., 2006). In many cases, traditional aging biomarker studies lack of a demographic and behavioral description of the populations used. Moreover, only one or few molecular parameters are eventually correlated with behavioral data.

The Mediterranean fruit fly *Ceratitis capitata* is a global orchard pest of great economic impact and one of the most studied species by biodemographers (Carey, 2011; Vaupel et al., 1998). However, most of the laboratory research in aging mechanisms has focused on genetically well-known organisms such as *D. melanogaster* (Helfand and Rogina, 2003) and *Caenorhabditis elegans* (Antebi, 2007). Pioneering studies in *C. capitata* described changes in main lipid classes during larval and adult stages (Madariaga et al., 1970). More recently, total lipid contents were shown to oscillate during *C. capitata* adult life, suggesting an endogenous regulation to maintain energetic balance (Nestel et al., 2005). In mosquitoes, cuticular lipids have been correlated with age and survival (Hugo et al., 2006). Age-dependent changes in dolichol levels in *Drosophila* showed interesting, though contradictory, results (Morris and Pullarkat, 1991; Parentini et al., 2005). These studies show the need to extend the analysis of changes in the overall lipid categories with age. Our aim was to condensate the quantitative changes in main categories of lipids during the adult life cycle in as few variables as possible. This was achieved using the multivariate approach called principal components analysis (PCA), that transforms data of correlated original variables into a smaller number of linear combinations called principal components (PCs) (Quinn and Keough,

Abbreviations: FSC, functional state component; PC, principal component; PCA, principal components analysis; TLC, thin layer chromatography.

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2002). In this analysis, most of the variation in the original variables is accounted for by the estimated first few new components (the PCs).

Several authors studied changes in mortality trajectories of fly populations subjected to environmental alterations, such as UV irradiation, hypergravity (Le Bourg, 1999), starvation, caloric restriction, density (Carey et al., 1995a; Gaskin et al., 2002), cold or heat stress (Helfand and Rogina, 2003), etc. Among environmental stressors, temperature has been associated with several traits such as longevity, body size, fecundity and fertility in *Drosophila* (Partridge et al., 1995; Norry and Loeschcke, 2002; Sisodia and Singh, 2002). *Drosophila* strains adapted to a given thermal regime showed lower longevity when reared under a different temperature (Partridge et al., 1995). Similar studies in *C. capitata* have shown a decrease in longevity when increasing the temperature from 25 to 30 °C (Shoukry and Hafez, 1979), and considerable differences in mortality have been reported among populations adapted to habitats with different mean temperature ranges and annual precipitations (Diamantidis et al., 2009).

In the present work we hypothesize that membrane and storage lipids reflect the functional state of an insect, and that they can be analyzed in a combinatorial way to statistically build novel indicators able to provide a grading score system to evaluate the age- and stress-dependent degree of senescence.

2. Materials and methods

2.1. Chemicals

1,2-dipalmitoyl-sn-glycerol and monomyristin were from Echelon Biosciences Inc. (Salt Lake City, UT, USA). Oleic acid, stearyl arachidate, methyl palmitate, cholesteryl palmitate, squalene, cardiolipin, 1-(3-*sn*-phosphatidyl)-*rac*-glycerol, L-α-phosphatidylcholine, L-α-phosphatidylinositol, L-α-phosphatidyl-L-serine, L-α-phosphatidylethanolamine, L-α-lisophosphatidylcholine, glyceryl trioleate and cholesterol were from Sigma-Aldrich Co. (St. Louis, MO, USA). Dolichol was from Avanti Polar Lipids Inc. (Alabaster, AL, USA). All solvents used were HPLC grade.

2.2. Insects

Standard populations of wild-type *C. capitata* (strain "Mendoza") larvae were reared in pumpkin-based medium (Pujol-Lereis et al., 2006). Flies were kept in a Conviron chamber CMP 3244, at 23 °C, 50–60% relative humidity, with a photoperiod of 16:8 light:dark. In all the experiments, adult flies less than 12 h old were collected, sexed under CO_2 and placed in flasks with free access to sucrose:dry yeast (3:1) and 1% agar as sources of food and water, respectively. Food and water were renewed every five days.

2.3. Lifespan assays

At the day of emergence (considered day 1), groups of 100 virgin flies, representing one laboratory population, were placed in 3.75 L flasks and maintained at 23 or 28 °C. Three replicas per sex were performed. Dead flies were counted and removed each day. We used the WinModest Program (Pletcher, 1999) to determine the mortality model that best fit the data, and to estimate the mortality parameters (see Section 2.7).

2.4. Spontaneous distribution of flies

We studied the spontaneous distribution of adult flies kept at 23 or 28 °C as a measure of their dispersal activity. Upper, middle and lower equal sections were marked in 0.5 L flasks (18 cm height) containing 40 flies (three replicas per sex). Flasks were placed at room temperature (21 °C) one hour before recording. The number of flies in each section of the flasks was counted at 10 AM of the days 2, 5, 10, 15, 20 and 30. We analyzed the age-dependent change in the proportion of flies in the lower section.

2.5. Negative geotaxis

Rapid iterative negative geotaxis (RING) assays were performed in our laboratory according to Gargano et al. (2005), and adapted to medfly. Flies of different ages (5, 15 and 30 days old) kept at 23 °C or 28 °C were tested to evaluate their ability to respond to a mechanical stimulus. Flies were placed in 0.5 L flasks, 40 individuals per flask and three flasks per sex. Ten hours before the trials, 10 flies per experimental unit were collected under CO_2 anesthesia, transferred to a 0.25 L test tube (28.5 cm height and 3.5 cm diameter) with 1% agar saturated with sucrose, and kept at room temperature overnight. This was done to let the flies recover from anesthesia. The trials were always started at 9 AM under the same illumination, at room temperature. Flies were forced to the bottom of the test tube by gentle tapping. As a consequence, the flies climbed up the side of the tube, and their position 10 s later was recorded with a digital camera (Sony DSC-W100). This was repeated 8 times for each sample, with

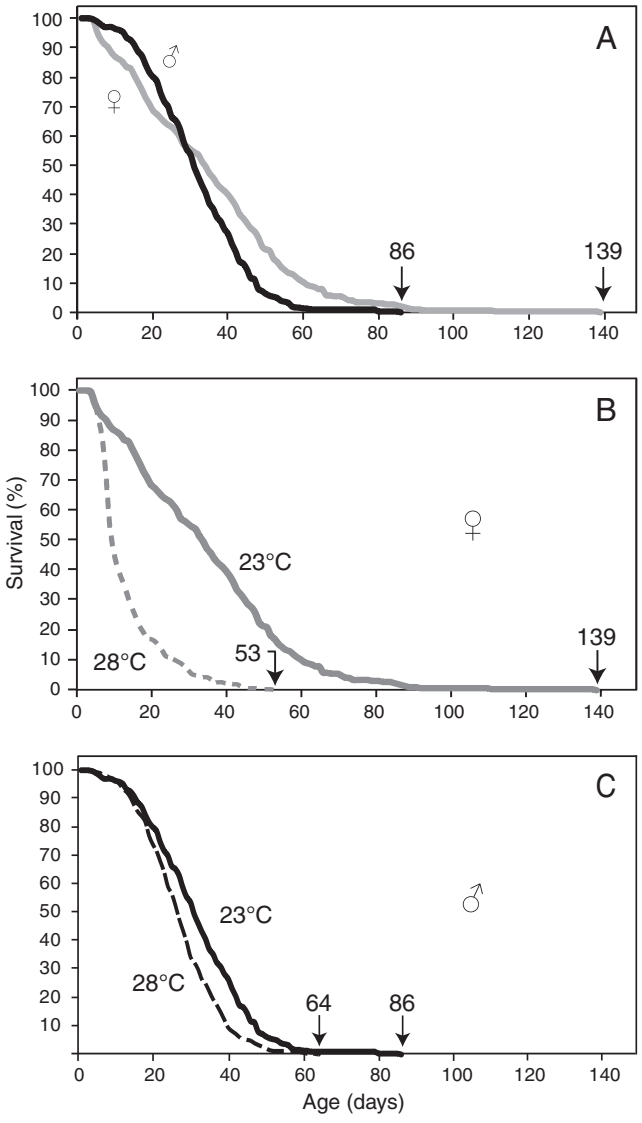

Fig. 1. Survival curves of *C. capitata* laboratory populations. (A) Females (gray) and males (black) at 23 °C. (B) Females under 23 °C (gray lines) and 28 °C (gray dashed lines). (C) Males under 23 °C (black lines) and 28 °C (black dashed lines). Arrows indicate maximum lifespan.

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Table 1
Demographic parameters of *C. capitata* laboratory populations.

Population	23 °C		28 °C	
	Females	Males [a]	Females [b]	Males
Logistic parameters				
Initial mortality rate (a)	0.0087	0.0022***	0.0002***	0.0015
Slope parameter (b)	0.06	0.13***	1.02***	0.18
Deceleration of mortality rate (s)	0.71	1.03	7.39***	1.28
Mean lifespan (days ± SEM)	35.1 ± 0.4	31.9 ± 0.3**	13.8 ± 1.3***	27.9 ± 2.2
Maximum lifespan (days)	139	86	53	64

** $p<0.01$.
*** $p<0.001$.
[a] Asterisks indicate significant differences between sexes at 23 °C.
[b] Asterisks indicate significant differences between temperatures.

exactly 2 min intervals. The distance climbed by the flies was measured using the Scion Image 4.0 software (Scion Corporation). For each sample, the average distances from the 3rd to the 7th repetition were compiled.

2.6. Lipid extraction, separation and detection

Lipids were extracted from whole bodies of 5, 10, 15, 20 and 30 day-old populations kept at 23 °C, body parts (head, thorax or abdomen) of 5, 15, 30 and 40 day-old populations kept at 23 °C, and body parts of 5, 15 and 30 day-old populations kept at 28 °C. At least three replicas per sex and age were carried out (except for 23 °C populations of 40 days: two replicas). Samples were homogenized in acetone and centrifuged at 7000 *g* for 20 min. The acetone supernatant was transferred and dried under a nitrogen flow. The pellet was re-extracted with chloroform: methanol (3:2), centrifuged at 7000 *g* for 20 min, and the supernatant was mixed with the acetone extract and dried. Total lipids were then solubilized and washed according to a modified Folch's method (Quesada-Allué and Belocopitow, 1978; Folch et al., 1957). In a preliminary assay, the lipid extracts were fractionated into the various classes of lipids by absorption chromatography in silicic acid columns according to Kates (1986). This silicic acid low pressure chromatography fractionation, previous to planar thin layer chromatography (TLC), was found to be unnecessary for our analytical purposes; whereas classical HPLC separation was time-consuming and not practical because of the large number of samples. 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, three-solvent system (I) as follows: (i) hexane 17.5 cm from the origin (f.o.); (ii) toluene 17.5 cm f.o.; and (iii) hexane: diethyl ether: acetic acid (70:30:1) 12 cm f.o. (Pappas et al., 2002). Neutral lipid classes were visualized by charring with a ferric chloride/sulfuric acid solution (see a typical chromatogram in Supplementary Fig. S1, and R_{chol} in Supplementary Table S1). To separate polar lipids, plates were developed with a one-dimensional, two-solvent system (II) 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. (Sterin-Speziale et al., 1992). 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 (see a typical chromatogram in Supplementary Fig. S2, and R_{PC} in Supplementary Table S1). Neutral and polar lipid standards were separated by TLC together with the samples. The method was calibrated in order to avoid saturation of the silica gel for any of the major lipid classes. Densitometry of lipid classes was performed using ImageJ/Fiji software, and the area under the peak for each spot was used to quantify in terms of arbitrary units.

2.7. Statistical analyses

Parametric mortality models (Gompertz, Gompertz-Makeham, Logistic, Logistic-Makeham) were fitted to data by maximum likelihood using the software WinModest Version 1.0.2 (Pletcher, 1999). Likelihood ratio tests were used to determine the mortality model that best fit the data (Pletcher, 1999). In all the populations analyzed, the best fitting model was the Logistic ($\mu_x = ae^{bx}[1+(as/b)$

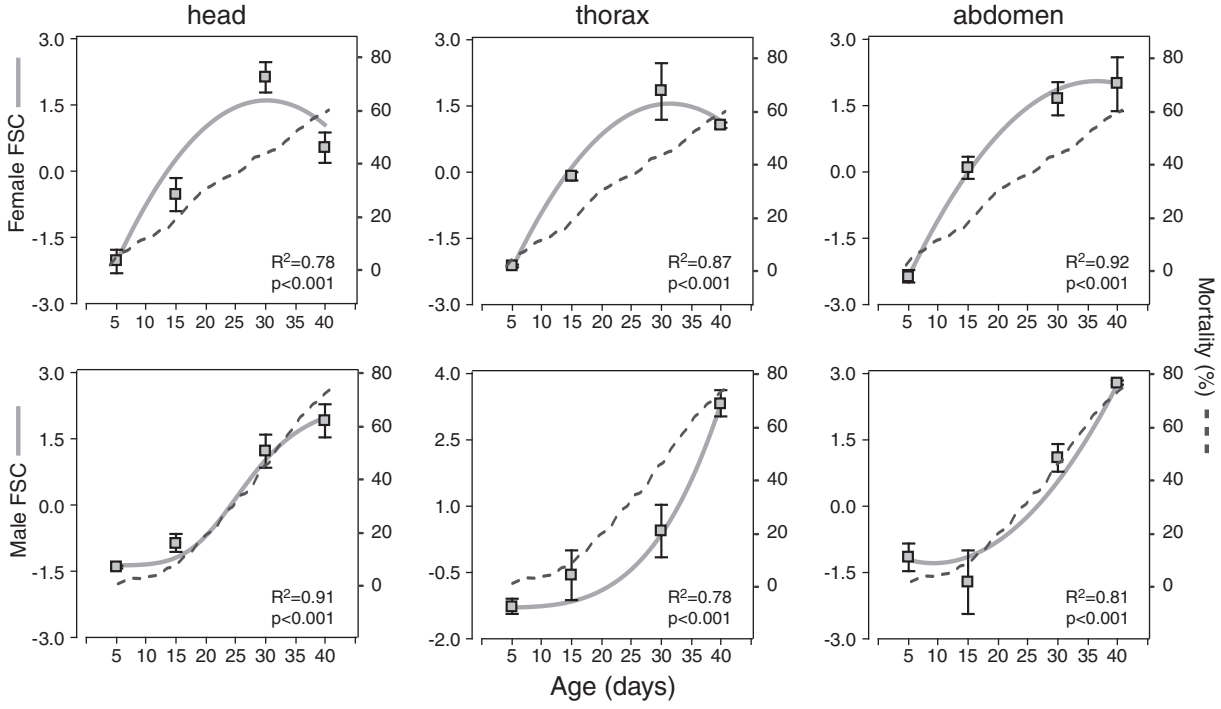

Fig. 2. Changes in body-part lipid patterns with age at 23 °C. Relationship between chronological age and FSCs of reduced-PCAs. Curves were fitted to polynomial or sigmoidal regressions (gray lines). Mortality curves are overlaid (black dashed lines).

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$(e^{bx}-1)]^{-1}$), where μ_x is the age-specific mortality at age x, a is the initial mortality rate, b is the age-dependent increase in mortality rate (slope parameter), and s is the deceleration of the mortality rate at advanced ages. The Logistic mortality parameters estimated for each population using WinModest were compared by likelihood ratio tests (Pletcher, 1999).

Principal components analyses (PCAs), analyses of variance (ANOVAs), Student's *t*-tests and correlation analyses were done using the Infostat 2010 Software (UNC, Córdoba, Argentina).

We used PCA to reveal patterns of change in the data. PCA reduces many variables (the lipid categories in our study) to a smaller number of new variables called principal components (PCs) (Quinn and Keough, 2002). We carried out PCAs using the densitometric values of the lipid categories at different ages. PCAs were based on correlation matrices, and the minimum eigenvalue was set at 1. Separate analyses were done for each sex, and for whole body, head, thorax and abdomen. We selected those PCs that showed significant regression on age (see Supplementary Fig. S3 for a comparison among the first three PCs of whole bodies). If more than one PC seemed to correlate with age for the same sex and body part, we chose the PC that explained the largest percentage of the total variance. As a result, we kept PC1 for the analysis of the lipid pattern of female heads and PC2 for the rest of the groups (these are the FSCs). The PCA output includes the PCs/FSCs scores for the samples introduced in the analysis (23 °C samples), the eigenvectors and the loadings. The loadings of the FSCs (Supplementary Table S2 and Table 2) are the correlations of each variable on each FSC. Therefore, lipid categories with higher loadings (either positive or negative) are more closely related to the patterns revealed by the FSCs. FSC scores for the 28 °C samples were calculated using the eigenvectors of the reduced PCAs, and the densitometric values of the lipid categories at 28 °C.

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

3. Results

3.1. Demographic characterization of C. capitata laboratory populations

It has been previously shown that *C. capitata* populations have a peculiar pattern of age-specific mortality that differs between females and males (Carey et al., 1995b). Under our experimental standard conditions (23 °C), the survival curves of the Argentinean strain "Mendoza" followed a similar pattern, with a crossover at around 30 days of age (Fig. 1A). We then determined the best-fit mortality model by using maximum likelihood methods (Pletcher, 1999; see Section 2.7). For both sexes, the Logistic model, characterized by the parameters a, b and s, was the best for describing the data ($p<0.0001$). The initial mortality rate, a, was significantly higher in females, whereas the age-dependent increase in mortality rate (b, hereafter called slope parameter) was significantly higher in males (Table 1, 23 °C), in agreement with the crossover of the survival curves in Fig. 1A. The s parameter, which describes the deceleration of the mortality rate at advanced ages, was not significantly different between sexes (Table 1). Mean and maximum lifespan were higher for females than males (Fig. 1A and Table 1, 23 °C).

3.2. Age-related indices built from lipid profiles

To evaluate overall age-dependent changes and establish a proof of concept, we first analyzed whole-body lipid patterns. Since sex-biases in mortality patterns are thought to depend on physiological, reproductive and behavioral singularities (Carey et al., 1995b; Carey, 1997), we studied females and males separately (see Section 2.6). Polar and neutral lipids of different ages were analyzed by TLC. Twenty-six main distinct categories (Supplementary Table S1) were separated and quantified by densitometry. We confirmed that triglycerides are the main lipid class in *C. capitata* lipids (see lipid profile of male thoraces in Supplementary Fig. S1) and variation in their levels reflect changes in total lipid contents. Multivariate analyses of the lipid categories were performed to find new derived variables that could summarize all this information, and reflect the functional state of the flies. Lipid patterns were examined by standardized PCA. Most important, no data on the age of the flies were included. We found lipid-dependent PCs that changed significantly with age, and we selected them as functional state components (FSCs) (i.e. PC2 in Supplementary Fig. S3; see loadings in Supplementary Table S2). Then, as whole-body analyses probably compensate differences in the lipid patterns among body parts, we analyzed lipid extracts from head (mostly brain), thorax (mostly muscles) and abdomen, at different ages. Examples of separation of neutral lipids of male thoraces and polar lipids of male heads are shown in Supplementary Fig. S1 and S2, respectively. As expected, a preliminary analysis showed that changes in each lipid category depended on the body part. For

Table 2
Reduced principal component analysis (PCA) loadings obtained for body-part FSCs.

Functional state component loadings						
Lipid identity	Head		Thorax		Abdomen	
	Females	Males	Females	Males	Females	Males
Neutral lipids						
Hydrocarbons	0.62	−0.26	0.53	−0.45	0.13	−0.47
Squalene	0.74	−0.43	0.87	−0.39	0.24	0.42
Sterol esters	0.64	0.69	0.37	0.66	−0.78	0.37
Wax esters	0.89	0.76	0.84	−0.16	−0.38	0.20
Fatty acid acyl esters	0.84	0.43	0.44	−0.59	0.52	0.01
Triglycerides	0.26	−0.33	−0.20	−0.57	0.21	−0.83
NL7[a]	−0.70	−0.24	−0.85	−0.73	−0.65	−0.49
NL9[a]	−0.63	−0.59	−0.80	−0.89	−0.97	−0.40
Sterols	−0.27	0.17	−0.20	0.23	−0.59	−0.41
1,2-diacylglycerol	−0.44	0.13	−0.18	−0.06	−0.60	−0.43
Monoglycerides-1	−0.56	0.06	−0.12	−0.20	−0.43	−0.28
Polar lipids						
Phosphatidylserine	0.76	0.23	−0.01	−0.36	0.65	0.82
Phosphatidylinositol	0.75	−0.05	0.12	−0.49	0.47	0.77

NL = neutral lipid.
[a] Not identified.

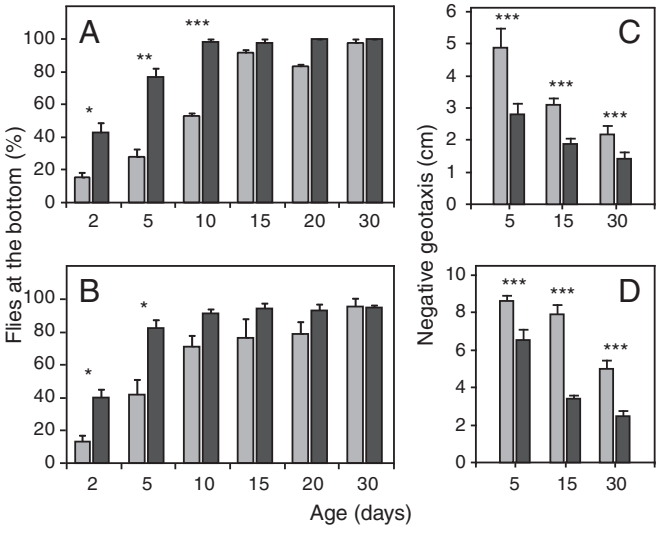

Fig. 3. Comparison of behavioral parameters between *C. capitata* laboratory populations maintained under 23 °C (light gray bars) or 28 °C (dark gray bars). Spontaneous distribution of females (A) and males (B) expressed as the percentage of flies in the bottom section of the flasks. Negative geotaxis performance of females (C) and males (D). Statistics: A and B, *t*-test with Bonferroni correction; C, two-way ANOVA, no interaction; D, two-way ANOVA and simple effect test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

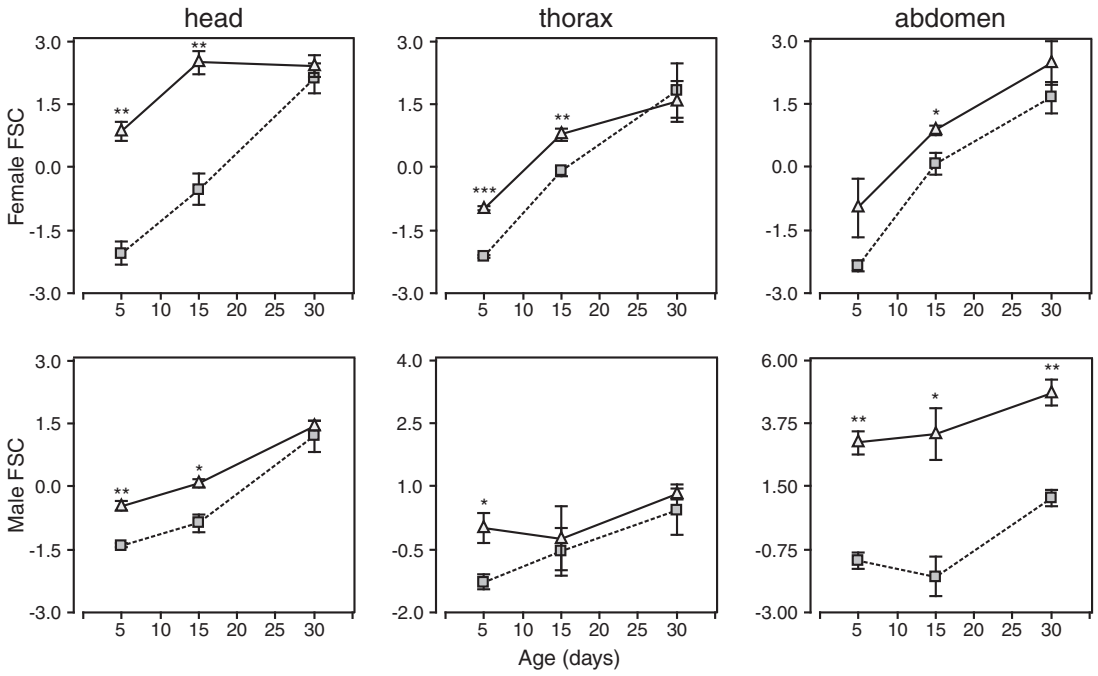

Fig. 4. Comparison of lipid patterns between populations maintained at 23 °C (solid squares) and 28 °C (open triangles). Relationship between chronological age and FSCs of reduced-PCAs. Significant differences between temperatures (*t*-test): *p<0.05, **p<0.01, ***p<0.001.

example, sterol esters showed an increase with age both in males and females (Supplementary Fig. S4A, D–F), with the exception of abdomen and 40-day-old thorax in females (Supplementary Fig. S4B and C). Other lipid classes showed more complex profiles depending on the body-part and sex. Supplementary Fig. S4I,L shows that phosphatidylserine seemed to increase with age only in abdomens, with the exception of 40 day-old females, and decreased with age only in female thoraces (Supplementary Fig. S4H). Therefore, for each body part and sex, a standardized PCA was performed. We obtained FSCs with significant non-linear regression on age (Supplementary Fig. S5 and Supplementary Table S2).

To further refine and increase the robustness of the analyses, our next step was to eliminate the variables with little contribution to the FSCs, and carry out reduced PCAs. Thus, PCAs were repeated using only the lipid categories that exhibited absolute values of loadings higher than 0.45 in at least two FSCs of the non-reduced analyses (Supplementary Table S2). The curves obtained gave better regression of the FSCs on age (Fig. 2; see Table 2 for FSCs loadings), so we kept these FSCs as the definitive FSC indices. We observed that the temporal variation of the lipid patterns in head, thorax and abdomen of females followed a similar polynomial-shaped trend (Fig. 2), with larger differences between 5 and 30 days old and smaller differences at older ages. In males, body-part FSCs followed a sigmoidal-shaped trend (Fig. 2), with small differences at young ages, and larger differences after day 15. These results were consistent with a higher initial mortality rate in female populations, and a higher slope parameter in male populations (Fig. 2 and Table 1). The similarity between the age-dependent trends in males' FSCs and mortality is striking (Fig. 2).

The contribution of each lipid class was different depending on the body part, as deduced from the FSC loadings (Table 2). PCA biplots were constructed to visualize the relationship between the lipid categories, the PCs and the samples. Head-PCA biplots show, as an example, that samples with similar physiological state became clustered, and that these groups were consistent with the chronological age of the individuals, not previously included in the analyses (Supplementary Fig. S6).

Triglycerides' loadings were different in head, thorax and abdomen of both sexes (Table 2). NL7 and NL9 had a great contribution in most FSCs (Table 2). They both decrease when the FSCs increase, which implies that they were in a smaller proportion in individuals with a worse functional state. NL7 was tentatively identified as triglycerides with hydroxylated fatty acid chains, but the identity has not been confirmed yet. NL9 behaved in several chromatographic systems as a long chain isoprenol (dolichol), but preliminary mass-spectra analyses were unable to identify isoprene groups.

Wax esters were important in head-FSCs (absolute value of loadings > 0.45: females, 0.89; males, 0.76) but less important in abdomen-FSCs (absolute value of loadings < 0.45: females, −0.38; males, 0.20). As FSCs increased while functional state decreased, then wax ester levels were higher (positive loading) in heads of females with a worse functional state (old flies at 23 °C; see Table 2 and Supplementary Fig. S6).

3.3. Demographic and behavioral parameters under mild thermal stress

Adult populations were kept at 28 °C, and demographic parameters were calculated and compared to those obtained at 23 °C. Female populations were highly affected by temperature, as showed by a 60% decrease in their mean lifespan (Table 1), and significant changes in all the parameters of the Logistic mortality model, with an important increase in the slope parameter (Fig. 1B and Table 1). Males were not significantly affected in their survival at 28 °C, despite a 26% decrease in their maximum lifespan (Fig. 1C and Table 1).

To have an insight on the age- and stress-dependent neuromotor output, behavioral parameters of both control (23 °C) and stressed (28 °C) populations were compared. We first studied the spontaneous distribution of the flies within flasks, as a measure of their dispersal activity (see Section 2.4). This behavior has been associated to insects' internal physiological state and local environment (Simon et al., 2011). Populations kept at 23 °C showed an age-dependent increase of the percentage of insects detected at the bottom of the flasks (Fig. 3A and B; non-linear regression, p<0.0001). When flies were kept at 28 °C, the percentage of individuals at the bottom was higher at younger ages, and tended to become equal to controls with increasing age (Fig. 3A and B). This was attributed to stress-dependent changes in the behavior of the flies. To further evaluate the age-related

locomotor impairment in response to an active physical stimulus, rapid iterative negative geotaxis assays were performed (Rhodenizer et al., 2008). As previously observed for *D. melanogaster* (Iliadi and Boulianne, 2010), adult flies of both sexes showed a significant decline in the performance of this behavior with age (Fig. 3C and D; $p<0.0001$). When kept at 28 °C, the performance was even worse than 23 °C controls at all ages (Fig. 3C and D). Although the distribution of flies was not significantly different between temperatures at 15 and 30 days old (Fig. 3A and B), negative geotaxis exhibited a locomotor impairment under 28 °C at those ages (Fig. 3C and D). This confirmed the necessity to test more than one behavioral parameter when studying the functional state of individuals.

3.4. FSCs obtained at 23 °C were able to reflect the decline in functional state under 28 °C

To test if the definitive FSC indices could reflect both the age- and stress-dependent decline in functional state deduced from the behavioral tests, body-part lipid profiles of populations maintained at 28 °C were analyzed, and FSC scores for the 28 °C samples were calculated (see Section 2.7). Head-FSC scores for young females (5 and 15 days old) kept at 28 °C were higher than for 23 °C controls of the same chronological age (Fig. 4), and similar to those for old 23 °C flies (30 days old). Analogous results were obtained for thorax and abdomen FSCs in females, though differences between temperatures were smaller (Fig. 4). Males also showed differences in head-FSC scores between young flies at 28 °C and 23 °C (Fig. 4), whereas thorax-FSC scores were less affected, with slight differences only for 5 day old flies (Fig. 4). The abdomen-FSC was highly affected in 28 °C males comparing with 23 °C males at all ages (Fig. 4).

4. Discussion

As a first step toward obtaining good indicators of functional senescence in the medfly, *C. capitata*, we studied changes in the lipid profile of populations with age, and under thermal stress. Demographic and behavioral tests were simultaneously performed, assuming that they reflect the functional output of the flies. We here demonstrated that the lipid-dependent PCs of PCAs that we called FSCs were able to discriminate both age- and temperature-dependent changes in the functional state of flies, in agreement with the demographic data and behavioral tests.

The study of demographic parameters of virgin flies under standard conditions (23 °C) showed a crossover of female and male survival curves (Fig. 1A) apparently typical for this species, as previously described by Carey et al. (1995b). These sex-biases have been previously associated with the constitutional endowment of flies, which determines their ability to resist unfavorable situations, the reproductive biology and the behavioral predispositions (Carey et al., 1995b). Therefore, subsequent studies were performed in males and females separately. Since it is believed that the expansion of the medfly in South America was the product of few events of invasion, our results demonstrate that the main demographic parameters of the species in the northern hemisphere were maintained.

Changes in the metabolism of certain lipids have been previously associated with the aging process in different animal models. Lifespan extension in flies, worms and mice through signal reduction of the insulin and insulin-like growth factor signaling pathways has been correlated with fat metabolism and fat tissue signaling pathways (Piper et al., 2008). Comparison of hepatic gene expression patterns of long-lived Snell dwarf mice and control mice showed changes in key transcripts involved in cholesterol biosynthesis, fatty acid metabolism and lipoprotein homeostasis (Boylston et al., 2004).

Here, we analyzed patterns of change in twenty-six lipid classes of fly populations at different chronological ages and under thermal stress conditions. After a preliminary approach using whole bodies (Supplementary Fig. S3 and Supplementary Table S2), we focused on the study of the lipid profiles in head (mostly neural tissue), thorax (muscle and thoracic ganglia) and abdomen. Some of the lipid classes tended to change with age (e.g., male thorax sterol esters, abdomen NL9), with stress (e.g., head free fatty acids, head diacylglycerides and most of the phospholipids), or with both age and stress (e.g., female abdomen sterol esters, head NL9 and thorax monoglycerides). However, none of these lipid classes could significantly be used as a single functional senescence indicator. Multivariate principal component analyses were performed to disclose patterns of change among the lipid classes and summarize them in a small number of new variables that could be used as robust functional state indices. Using the data from standard 23 °C samples, PCs that significantly correlated with age were obtained for each body part and sex (Supplementary Fig. S5). Moreover, in a second analytical step, reducing the PCAs we obtained better regressions of the PCs that we called FSCs (functional state components) on age (Fig. 2). As pointed out in Section 3.2, the differences in the temporal variation of FSCs of males and females were consistent with mortality curves and demographic parameters (Fig. 2 and Table 1).

The contribution of each lipid class to each FSC was indicated by the loadings in Table 2. In medfly, most of the fatty acids from glycerides and phospholipids are unsaturated (Pagani et al., 1980), thus favorable to membrane fluidity. NL7 was tentatively identified as triglycerides with hydroxylated fatty acid chains, and showed high negative correlations with most of the FSCs (Table 2). A decrease of hydroxylated triacylglycerols might eventually contribute to a decrease in membrane fluidity.

Cuticle wax esters are important for restricting water loss in insects, and thus prevent tissue stress (Gibbs, 2002). We observed that wax esters had a high positive correlation with head-FSCs of both sexes and thorax-FSC of females (higher than 0.75, Table 2). This is in agreement with age-dependent changes previously reported in the composition of cuticular lipids in insects (Hugo et al., 2006). In honeybees, the synthesis of cuticular waxes depends on the age of the insects, the season of the year, and the body part (Blomquist and Chu, 1980).

The positive contribution of sterol esters (cholesterol esters and others) to the FSCs (see Table 2 and Supplementary Table S2) probably reflects changes in the overall sterol metabolism and composition of tissue membranes, since adult fat body is scarce. In female abdomens, negative loadings for the sterols and sterol esters (Table 2) indicate a decrease in these lipid classes with functional state. As ovaries in adult females occupy most of their abdomen, the decrease of sterol metabolites may be due to the previously reported decrease in egg production as a function of age (Zhao et al., 2008) and stress (Rinehart et al., 2000). Medfly cholesterol metabolism, as in other insects, is totally dependent on dietary sterols since they cannot synthesize the steroid nucleus (Svoboda and Feldlaufer, 1991). Thus, in laboratory conditions with a controlled diet, age- or stress-dependent changes should be due to alterations in the sterol conversion processes.

The differences among body parts reported here could be related to the known differential sensitivity of tissues to aging factors in other animals as well as humans (Simm and Johnson, 2010). We previously observed in *C. capitata* that brain ganglia and abdominal longitudinal muscles seem to be the most sensitive to oxidative stress induced by photoinsecticides (Pujol-Lereis et al., 2010; Berni et al., 2003). Girardot et al. (2006) described *Drosophila* thorax-specific changes in the regulation of mitochondrial genes, the JNK pathway and the proteasome subunits, thus suggesting a particular sensitivity of muscles to aging. They also found a selective downregulation of synaptic transmission genes in *Drosophila* heads. Using the same model organism, Zhan et al. (2007) investigated changes in the transcriptome over six age-points and seven tissues, including brain, thoracic muscle, fat body, gut, malpighian tubules, accessory glands

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and testis. They found that age-related genes are generally regulated in a tissue-specific manner, and with different temporal patterns, concluding that tissues with the same chronological age could have different physiological ages (Zhan et al., 2007).

Since our lipid-dependent indices were able to reveal age-dependent functional states, we hypothesized that they would also reflect changes in the composition of cellular membranes and storage lipids under different types of stress. In order to elucidate which of the FSC indices best reflect the functional state of the flies, we stressed fly populations under 28 °C. We expected the FSCs obtained at 23 °C, which reflect normal physiological changes, to explain variations in the lipid profile of flies reared at 28 °C, a mild heat stress for the medfly. The analysis of lipids clearly showed that young stressed flies had a lipid pattern similar to old control flies (Fig. 4), suggesting that the FSCs mainly reflect the homeostatic equilibrium, and therefore the physiological age attained by flies under certain conditions, instead of their chronological age.

Thermal heat stress in insects produces alterations in proteins, DNA, RNA, lipids and carbohydrates, and also causes problems in the interaction of cells and the integration of physiological processes (Denlinger and Yocum, 1999). Under thermal stress, the fluidity of cellular membranes is partially maintained by changes in the composition of membrane phospholipids' fatty acids (Overgaard et al., 2008). Higher cholesterol concentrations have also been associated to heat response in flight muscle mitochondria of the grasshopper *Schistocerca gregaria* (Downer and Kallapur, 1981).

It has been postulated that death caused by high temperature stress in multicellular organisms is not due to massive cell death, but to the failure of a critical tissue (Denlinger and Yocum, 1999). Despite the similarity of the temporal trends of the FSCs for each sex, there was a different sensitivity of the body parts to the thermal stress (Fig. 4). Our results show that a shift to 28 °C of a population of *C. capitata* females maintained at 23 °C caused a decrease in the behavioral output of the central nervous system (distribution and negative geotaxis tests, Fig. 3A and C) and an increase in their mortality (Fig. 1B and Table 1). This could be correlated with the changes observed in the corresponding females' FSCs, which showed that lipid profiles in young stressed populations where primarily affected in head, and to a lesser extent in thorax and abdomen. In males, the changes produced under thermal stress caused statistically significant but smaller differences in head lipid profiles. This may reflect an increase in functional senescence below an unknown physiological threshold since no increase in mortality was observed (Fig. 1C and Table 1), even though their behavior was altered (Fig. 3B and D). Lesser differences in thorax lipid profiles of both sexes were in concordance to previous studies that demonstrated that insect thoracic muscles can withstand high temperatures (Heinrich, 1974; Loli and Bicudo, 2005). Major temperature-dependent changes were observed for the males' abdomen-FSC at 28 °C (Fig. 4) mainly due to the contribution of triglycerides, phosphatidylserine and phosphatidylinositol (Table 2). Changes in membrane phospholipids and mobilization of lipid reserves have been previously linked to homeostasis maintenance under stress (Neven, 2000; Arrese and Soulages, 2010). As males' mortality was not significantly affected at 28 °C, male's abdomen-FSC may evince the necessary changes to maintain homeostasis rather than the functional output of flies.

Our results indicate that the lipid profiles of heads seem to better reflect the behavioral and demographic changes caused by age and stress. The magnitude of the temperature-dependent differences in the head-FSCs (Fig. 4) could explain the decrease in the behavioral output for both sexes, as well as the increase in females' mortality, where differences in FSCs were larger. Therefore, head-FSCs appear to be novel reliable indicators of functional senescence in flies. We believe these novel indices establish a proof of concept in reference to the lipid profile changes during senescence and stress. The study of *C. capitata* populations under other types of stress, and of normal and long-lived strains of *Drosophila*, will further test the validity of the indicators here reported. Since lipid profiles also depend on the fly diet composition, wild populations would be more difficult to study, but overall dietary profiles can be established in a given orchard, fruit ecosystem or medfly mass-rearing facility.

Our findings open up the possibility of new approaches in the study of senescence using lipid profiles, since we here demonstrated that they change with functional state. Further studies are needed to understand these changes and their relationship with the aging process. Since our first aim was to condense the data in indices able to reflect the functional senescence status, no studies on causality or mechanisms were carried out yet.

We believe that our results also open new approaches to the study of aging in other arthropods, and eventually other organisms.

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