Journal of Insect Physiology 87 (2016) 53–62

Contents lists available at ScienceDirect

Journal of Insect Physiology

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

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

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ARTICLE INFO

Article history:
Received 17 August 2015
Received in revised form 21 December 2015
Accepted 6 February 2016
Available online 8 February 2016

Keywords:
Chill-coma recovery
Chilling-injury
Medfly
Lipid profiles
Fatty acid composition
Sterol esters

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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http://dx.doi.org/10.1016/j.jinsphys.2016.02.002
0022-1910/

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