Variation in membrane composition associated with body mass and body temperature in tropical and North American bees

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"Ought we, for instance, to begin by discussing each separate species - man, lion, ox, and the like - taking each kind in hand independently of the rest, or ought we rather to deal first with the attributes which they have in common in virtue of some common element of their nature, and proceed from this as a basis for the consideration of them separately?" – Aristotle, *De partibus animalium.* Quoted in Harvey and Pagel (1991), p. 35.

- "All nature, from the smallest thing to the biggest, from a grain of sand to the sun, from the protista to man, is in a constant state of coming into being and going out of being, in a constant flux, in a ceaseless state of movement and change." – F. Engels, *Dialectics of Nature* (New York, NY: International Publishers, 1976), p.13.

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16:0 = palmitic acid

18:0 = stearic acid

18:1 (n-9) = oleic acid (omega-9)

18:2 (n-6) = linoleic acid (omega-6)

18:3 (n-3) = alpha-linolenic acid (omega-3)

22:6 (n-3) = docosahexaenoic acid (DHA, omega-3)

BMR = basal metabolic rate

MUFA = monounsaturated fatty acid

NEFA = non-esterified fatty acid

NL = neutral lipid

PI = peroxidation index

PIC = phylogenetically independent contrasts

PL = phospholipid

PUFA = polyunsaturated fatty acid

SE = standard error

SFA = saturated fatty acid

T_{th} = thoracic temperature

T_a = ambient temperature

T_{th}-T_a = thoracic temperature excess

UI = unsaturation index

Abstract

Membrane diversity associated with variation in body mass and body temperature of hymenopterans was investigated. Body mass of organisms affects most aspects of their biology, including physiological traits. One trait influenced by mass is metabolic rate, but the basis for its variation among organisms remains unclear. Recent work revealed that membrane phospholipid composition varies systematically with body mass: smaller vertebrates with higher mass-specific metabolic rates have more fluid membranes containing more long chains of polyunsaturated fatty acids (PUFA) and less monounsaturated fatty acids (MUFA). This "membrane pacemaker" theory of metabolism has recently been challenged by a lack of link between metabolic rate and membrane composition. To test this relationship, we have quantified the membrane lipid composition of 22 species of Panamanian orchid bees with a 22-fold range in mass. Results incorporating phylogenetic analysis show significant relationships for 18:1 (MUFA) and 18:3 (PUFA) with body mass that are consistent with the "pacemaker" theory, and unexpected relationships with saturated fatty acids (SFA). Moreover, changes in membrane fatty acid composition with temperature are a strategy in ectothermic poïkilotherms that is part of the "homeoviscous adaptation" model. Here, we report systematic variations in fatty acid composition linked with thoracic temperature excess in North American Hymenopterans, a novel discovery in these heterotherms. These findings are discussed in the context of diet, metabolism and lifespan.

Résumé

Nous avons étudié le lien entre la diversité membranaire et la variation en masse et température corporelle de divers hyménoptères. La masse des organismes affecte plusieurs aspects de leur biologie, dont certains traits physiologiques. L'un de ceux-ci est le taux métabolique, or, la base de sa variation entre organismes reste sujette à débat. Des travaux récents ont montré que la composition phospholipidique des membranes varie aussi systématiquement avec la masse : les petits vertébrés au taux métabolique spécifique plus élevé ont des membranes plus fluides, avec plus d'acides gras polyinsaturés (PUFA) et moins de monoinsaturés (MUFA). Cette théorie des membranes comme « rythmeur » du métabolisme a été récemment mise en doute par le manque de lien solide entre le taux métabolique et la composition membranaire. Afin de tester cette relation, nous avons étudié la composition des lipides membranaires chez 22 espèces d'abeilles des orchidées du Panama ayant une étendue de taille d'un facteur 22. Les résultats incluant une analyse phylogénétique montrent des relations significatives pour le 18:1 (MUFA) et le 18:3 (PUFA) avec la masse, en accord avec la théorie du « rythmeur » du métabolisme, et des relations inattendues au niveau des acides gras saturés (SFA). De plus, le modèle des adaptations homéovisqueuses prévoit des changements membranaires chez les ectothermes poïkilothermes comme stratégie pour le maintien de l'homéostasie cellulaire. Ici, nous avons observé des changements systématiques de la composition en acides gras en fonction de l'excès de température du thorax chez des hétérothermes, des Hyménoptères d'Amérique du Nord. Ces résultats sont discutés dans les contextes du métabolisme, de la longévité et de la diète.

CHAPTER ONE – General introduction

Biological membranes play important structural and functional roles in organisms. The diversity in membrane composition among species is remarkable, and its influence on the overall physiology of animals remains misunderstood. The objective of this thesis is to examine the diversity in muscle membrane composition in various insect species, and its relationship with variation in body mass and body temperature. This first chapter serves as a brief introduction to membranes, metabolism and temperature. In the second chapter, the relationship between body mass and thoracic membrane composition is studied in 22 species of orchid bees, and the implications for metabolic processes are discussed. In the third chapter, the diversity in thoracic membrane composition related to variation in thoracic temperature is investigated. Finally, in the fourth and final chapter, the results from the two data chapters are discussed and compared with the results found in vertebrates. The potential implications of metabolism, lifespan and diet are also discussed.

Cellular membranes are formed from pre-existing membranes, and due to this ubiquitous nature they can be thought of as "eternal structures" supporting life (Hulbert, 2003). Despite being only ~40Å thick, these assemblies of proteins and lipid molecules are responsible for the maintenance of the integrity of cells and organelles (von Heijne and Rees, 2008). The lipids and proteins that constitute this barrier are held together in a bilayer arrangement by non-covalent bonds. Membranes have conserved this bilayer structure for the past 3 to 3.5 billion years, although their specific lipid composition has changed radically throughout evolution (Barenholz, 2002). Lipids found in membranes consist of four types of phospholipids, glycolipids and cholesterol, all amphipathic molecules (possessing both hydrophilic and lipophilic properties). The polar head group is the hydrophilic part of the phospholipid and is thus positioned towards the surrounding aqueous medium, while the hydrophobic part consists of two fatty acyl chains that are positioned towards the inside of the bilayer. Fatty acids are carboxylic acids with a long hydrocarbon tail, and can be either saturated or unsaturated. Saturated fatty acids only possess single bonds and allow rotation around the carbon-carbon bond, while unsaturated fatty acids can have one (monounsaturates) or two to six double bonds (polyunsaturates). Double bonds constrain the rotation around the consecutive carbons, but provide a kink in the chain that can affect the surrounding lipids in the bilayer (Hulbert and Else, 1999). The hydrocarbon chain is typically composed of between 16 and 22 carbons, with an average length of 18 carbons. Fatty acid nomenclature typically refers to the number of carbons and number of double bonds as well as the location of the double bond. For example, oleic acid, 18:1 (n-9), is an 18 carbon monounsaturate and is classified as an omega-9 fatty acid (the first double bond is on the 9th carbon from the methyl end of the chain). The variability in fatty acids and polar head groups can generate thousands of different lipid species in the membrane, thus having important consequences on membrane-associated processes (von Heijne and Rees, 2008).

Body mass affects many aspects of an animal's biology. The scaling of biological traits with body mass has intrigued researchers for over a century, and many effects of body mass on animals remain misunderstood. As early as in 1883, Max Rubner proposed the surface hypothesis, following his observation that the metabolic rate of mammals did not increase proportionally with body mass, but instead with a 0.67 power corresponding to the scaling of surface area with changes in body mass. In 1932 Max Kleiber expanded the mammal species used and found that metabolic rate varied as the 0.75 power of mass, a relationship thereafter named Kleiber's law. The true nature of this relationship remains an active subject of research (West *et al.*, 1997; Darveau *et al.*, 2002; Banavar *et al.*, 2003; White and Seymour, 2005; Agutter and Tuszynski, 2011), nevertheless the consequence of this relationship is that mass-specific metabolic rate is inversely related to body mass: smaller animals have higher metabolic activity per gram of tissue (Schmidt-Nielsen, 1984). Despite these important differences, the relative contribution of various processes to basal metabolic activity in homologous tissues in animals is similar. Although these facts are well established in Biology, the proximal reason of why an animal has a certain metabolic rate remains misunderstood and subject to debate. Basal metabolic rate (BMR), the minimal energy expenditure of a homeotherm in a thermoneutral zone and in a post-prandial state measured as oxygen consumption, is principally linked to mitochondrial activity. In fact, non-mitochondrial processes account for 10% of the oxygen consumed, while ATP synthesis and proton leak in mitochondria account for 90% of oxygen consumption during BMR (Rolfe and Brown, 1997). Among the major processes contributing to mitochondrial oxygen consumption, many are linked to membranes. In fact, proton leak, Na^+/K^+ ATPase and Ca^{2+} ATPase are alone responsible for approximately half of the BMR. Other mechanisms such as protein synthesis or actomyosin ATPase are linked to membrane-bound structures. These facts place membranes in a very central position in the context of energy metabolism.

The first evidence of a link between membrane composition and metabolic rate comes from the work of Gudbjarnason *et al.* (1978), who showed a direct correlation between the heart rate of mammals (ranging from mice to whales) and the content in 22:6 (n-3) (DHA, a very polyunsaturated fatty acid) of their cardiac phospholipids. Moreover, a series of "species-crossover" experiments have demonstrated that the molecular activity of

Na^+/K^+ ATPase is strongly determined by local membrane lipid composition (Else and Wu, 1999; Wu and Hulbert, 2004). Therefore, because the nature of membrane lipids affects the local environment of the membrane and many metabolic processes are related in part to the membrane, changes in its composition among animals have been proposed as an explanation for the diversity in metabolic rate. This triggered the membrane pacemaker theory of metabolism (Hulbert and Else, 1999). However, these results have been recently challenged by a study for high or low metabolic rate on mice (Brzęk *et al.*, 2007), a study on 30 mammalian species (Valencak and Ruf, 2007), and a dietary study on hamsters (Pannorfi *et al.*, 2012). Thus, it appears that inter- and intra-specific studies do not yield the results predicted by the theory. The second chapter of this thesis therefore aims to examine this important theory of animal energetics by looking at the membrane composition in an invertebrate model within a fine phylogenetic context and a smaller body mass range.

Membrane structure and function is highly dependent on the dynamic state of lipids forming the biological membrane. As described earlier, the phospholipids are incredibly diverse and display different physical properties and behaviors. They are very sensitive to changes in the local physical environment such as variations in pressure, pH or temperature. In order to maintain cellular homeostasis, membrane lipid composition can be actively restructured in response to these environmental changes. This answer to environmental pressure in organisms that face variable environments or that have adapted to different conditions has been termed homeoviscous adaptation (Sinensky, 1974; Hazel and Williams, 1990). The third chapter of this thesis looks at the diversity in membrane composition for insect species with varying thoracic temperatures, in the context of homeoviscous adaptation. The most common response in poikilothermic organisms (with varying internal temperature) to a decrease in temperature is an increase in the fatty acid unsaturation of the membrane (Logue *et al.*, 2000). Cold-induced ordering of the membrane is thus countered by the increase in unsaturates, causing disorder to maintain membrane fluidity. This response is not only seen in animals, but is also known to occur in plants such as *Arabidopsis* (Uemura *et al.*, 1995), and in organisms living in very different environments. It also spans across various taxa, genera and species: remodelling of the membrane or adaptive changes in fatty acid composition are common in vertebrates and invertebrates. The numerous examples of changes in membrane composition include reptiles (Geiser and Learmonth, 1994; Seebacher *et al.*, 2009), fishes (Logue *et al.*, 2000; Guderley, 2004; Buckley, 2006; Kraffe *et al.*, 2007), mammals (Ruf and Arnold, 2008); but also flies (Overgaard *et al.*, 2005; MacMillan *et al.*, 2009), earthworms (Bindesbol *et al.*, 2009), springtails (Van Dooremalen *et al.*, 2011), and marine crabs (Cuculescu *et al.*, 1995; Cuculescu *et al.*, 1999). Despite the remarkable diversity in organisms and environments where membrane remodelling occurs, whether this adaptive response took place in heterothermic species such as bees and wasps has not been studied to date. The homeoviscous adaptation theory provides a framework to study the potential occurrence of changes in fatty acid composition in these flying insects, which is the objective of the third chapter of this thesis.

CHAPTER TWO – Membrane composition variation with body mass in flying insects: implications for metabolic diversity

Introduction

Membranes are essential components of life present in almost all organisms, from bacteria to eukaryotes. They have basic structural roles, acting as protective barriers and creating an internal environment for the cells and organelles through compartmentalization. They also have important functional roles in mediating transport, reception, transduction, attachment, catalysis or communication, and are therefore intrinsically linked to a variety of physiological processes. Due to their predominance and importance, recent research indicates that they could play key roles in explaining cellular bases of metabolic rate diversity, thermal physiology, aging or human diseases (reviewed by Hulbert and Else, 2005). Membrane composition, and most importantly the nature of the phospholipids forming the bilayer is thought to influence many mechanisms; from setting the pace of metabolic processes, to maintaining a suitable cellular environment when facing a wide range of temperatures, or influencing lifespan through peroxidation, and finally through diet and its consequence in the existence of a cluster of diseases. Despite its importance, membrane diversity and its implications remain misunderstood in many ways. This research investigates species diversity in muscle membrane composition in insect species, and its relationship with variation in body size.

Basal metabolic rate (BMR) varies widely among organisms, and the physiological basis of this variation remains a central theme in animal physiology. Researchers have tackled the problem using various approaches over the last three decades. When comparing a reptile and a mammal of the same body mass and at the same temperature, Brand and colleagues (1991) discovered that the latter had a basal metabolic rate 7-fold greater than the reptile. When investigating the underlying mechanism of this finding at the cellular level, they reported a much greater respiration rate of the liver mitochondria of the mammal and a higher proton permeability of the mitochondrial membrane. They found a strong correlation between this high permeability and the fatty acid composition of mitochondrial membrane phospholipids. Hulbert and Else (1999) further expanded this finding to a theory in which they suggested that membranes act as the "pacemakers" of metabolism. Evidence collected over the years suggests that BMR-related processes are linked directly or indirectly to membranes. In resting mammals, although most of the oxygen consumed is directed towards ATP production, mitochondrial proton leak also accounts for part of BMR (Rolfe and Brown, 1997; Hulbert and Else, 1999). Mammalian mitochondria are 4-5 fold more permeable to protons than reptilian mitochondria, and mitochondrial proton leak through mammalian membranes is estimated to account for 20-25% of the BMR (Brand *et al.*, 1991). Moreover, membrane-bound structures are involved in protein synthesis, proton and ion pumping or transfer, all of which are major ATP-consuming processes (Rolfe and Brown, 1997).

The physical and biochemical properties of membranes are both strongly influenced by the fatty acid composition of the lipid bilayer. As such, the presence of polyunsaturated fatty acids (PUFA, long hydrocarbon acyl chains with two or more double bonds) in the phospholipid fraction will generally help maintain greater membrane fluidity (Hazel, 1995; Yehuda *et al.*, 2002). Considering that the major cellular energy consuming processes are associated with membrane function, Hulbert and Else (1999) suggested that the wide variation in metabolic rate in animals may be linked to membrane composition. Thus, a single factor may be responsible for setting the pace of cells, where lipid acyl chains of membranes influence the molecular activity of membrane-bound proteins and therefore of membrane pump-leak cycles, acting as the "pacemaker" of metabolism. Their subsequent work showed that in mammals and birds, the degree of unsaturation of fatty acids in the membranes and body mass are negatively correlated. Specifically, as body mass increases, the unsaturation index (the number of double bonds per 100 acyl chains) decreases in these vertebrates. Moreover, mammals and birds of higher body mass have more oleic acid (18:1 n-9), a monounsaturated fatty acid (MUFA); while the amount of docosahexaenoic acid (DHA; 22:6 n-3), a polyunsaturated fatty acid decreases with animal size (Hulbert, 2003). The acyl composition of membrane bilayers therefore varies in a similar fashion to changes in BMR according to the well documented mouse-to-elephant curve (Kleiber, 1947; Schmidt-Nielsen, 1984), and their composition influences membrane-associated processes. Thus, it is proposed that the fatty acyl composition of membranes, and more importantly the balance between MUFA and PUFA, act as a "pacemaker" for metabolic activity.

The series of studies by Hulbert and Else has exploited variation in metabolic rate associated with body size. For over a century, biologists have been intrigued by the relationship between animal size and metabolic rate. The recent research on scaling has converged towards an allometric relationship between body mass and metabolic rate, but the details of this mathematical relationship and the opposing theories of allometric scaling are still subject to vigorous debate (Suarez *et al.*, 2004; Chaui-Berlinck, 2006; Agutter and Tuszynski, 2011). It is known, for instance, that the mass-specific metabolic rate is negatively correlated with body mass in a vast majority of animal species, and that those with larger body sizes have lower mass-specific metabolic rates (Childress and Somero, 1990). There is, however, only indirect evidence of correlated evolution of metabolic rate and membrane composition. For example, in mice artificially selected for high and low basal metabolic rate, no changes in membrane composition were observed (Brzęk *et al.*, 2007). In a study on hamsters exposed to a choice of thermal environments while fed SFA or PUFA-rich diets, Pannorfi *et al.* (2012) did not find the predictable changes in resting metabolic rate associated with a more polyunsaturated diet. Moreover, in a study on 30 mammalians species,Valencak and Ruf (2007) found no correlation between muscle phospholipid fatty acid composition and BMR. Their results showed that mammals with a high degree of membrane unsaturation did not have higher than expected BMRs, questioning the "membrane pacemaker" theory. Thus, in order to further pursue this potentially central theory of animal energetics, the association between metabolic rate and membrane composition needs to be scrutinized.

To improve our understanding of the importance of membranes and their possible role as pacemakers of metabolism, a model that would specifically link species metabolic rate and membrane properties of a specific tissue would be beneficial. Flying insects sustain some of the highest mass-specific oxygen consumption rates (VO_2), of any animals, and it is estimated that the thoracic flight muscle accounts for more than 90% of this metabolic rate (Staples and Suarez, 1997). Bees are myogenic flyers, with stretch-activated wing muscles that consist of a single fiber type and that are fueled mainly by carbohydrate oxidation (Suarez *et al.*, 2005; Woods *et al.*, 2005). In a recent study on Panamanian orchid bees (Darveau *et al.*, 2005), body mass was shown to strongly affect flight mass-specific metabolic rates, and this relationship could be explained by wing morphology and kinematics. Given that metabolic energy production during hovering flight is almost entirely supported by one tissue, the flight muscle, the links between tissue properties and metabolic rate diversity can be assessed within a fine phylogenetic context and body mass range.

Question and predictions

To investigate these aspects of membrane physiology, this study will address the following question. In order to test the "membrane pacemaker" theory of metabolism, **what is the membrane lipid composition of the orchid bee flight muscle and how does it vary with body mass?** If this invertebrate model follows a trend that is similar to mammals and birds, we would predict that bee species with lower body-masses (higher mass-specific metabolic rates) will have a higher proportion of PUFA in their muscle membranes and lower MUFA. Consequently, bigger species will have membranes containing less PUFA and more MUFA.

Materials and methods

Field sampling of orchid bees

Individuals from 22 species of Euglossine bees were sampled in May and June 2009 using chemical baits on Barro Colorado Island and Cerro Campana located in Panama. A total of 188 individuals were sampled in the field and transported to the laboratory in transparent 50mL tubes maintained on ice. Figure 2.1 illustrates the contrast in size among Euglossine bees. Body masses of the collected bees ranged from 49.6 to 1080mg (detailed in Table 2.1). Upon return to laboratory, animals were identified, dissected, and body parts were weighted, freeze-dried and placed in 1.5mL CryoTube vials for transport back to Canada where they were stored in a -80°C freezer.

Lipid analysis

Whole-thorax lipids were first extracted using a 2:1 mixture of chloroform and methanol (Folch *et al.*, 1957) and homogenized with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland). A fraction of the homogenate (5mL) was taken for immediate analysis; while the rest was stored in glass tubes at -20C. Samples were then centrifuged (10mins at 3000 RPM) and filtered in three successive steps; water was removed by adding 2.5 mL of KCl (33 mM) and the aqueous phase was eliminated with a water-powered vacuum pump. After evaporation on a Büchi RE 121 rotary evaporator from Brinkman Instruments Inc. (Westbury, NY, USA), samples were re-suspended in 6mL of a 3:2 (v:v) mix of hexane and isopropanol; then stored in the freezer at -20°C until lipid separation was performed.

Separation of lipids was done following a protocol described in Magnoni and Weber (2007). Lipid fractions of neutral lipids (NL), non-esterified fatty acids (NEFA) and phospholipids (PL) were separated by filtration on 1ml/100mg Supelclean solid-phase extraction tubes (Sigma, St-Louis, Missouri, USA). Heptadecanoic acid (17:0; 30mg/100ml hexane) was added to each fraction as an internal standard because preliminary experiments had shown that this fatty acid (FA) is absent from NEFA, NL and PL fractions of bee tissues. Following the separation, the NEFA fraction was methylated using a mix of methanol, dimethoxypropane and concentrated HCl (1M). The NL and PL fractions were transesterified using 2mL of acetyl chloride (1M) in methanol and heated to 90°C for 2 hours. The three fractions were then evaporated, re-dissolved in 50µL of isooctane and transferred to gas chromatograph autosampler vials.

The fractions were analyzed on an Agilent technologies HP 6890 series gas chromatograph system equipped with a flame-ionization detector and a 60 m DB-23 fused silica capillary column (internal diameter: 0.25mm and 0.25µm film; J&W Scientific, Folsom, CA, USA). The temperature program of the column consisted of 1 min at 130°C after injection, then an increase of 6.5°C.min^{-1} to a temperature of 170°C. Immediately after, an increase of 2.75°C.min^{-1} was applied to reach 215°C maintained during 12min. The temperature was then raised at a rate of 40°C.min^{-1} to reach 230°C, a temperature which was subsequently maintained during 3 min. The total run time for each sample was of 38.89 min. Individual fatty acids were identified by determining exact retention time with authentic standards (Supelco, Bellefonte, PA, USA). The relative abundance of individual fatty acids was measured using the surface area under the peak and correcting for the internal standard (17:0) in the ChemStation software environment (Agilent Technologies, Wilmington, DE, USA).

Data analysis

All statistical analyses were performed using the Systat 13 software. The relative abundance of each fatty acid (expressed in %) in the PL, NL and NEFA fractions was plotted against species body mass. All values are presented as mean ± SE. Dependent and independent variables were first tested for normality using the Shapiro-Wilk test. We also calculated two indices related to the level of unsaturation of the membrane, namely unsaturation index (UI) and peroxidation index (PI) using the definitions found in (Hulbert *et* *al.*, 2007). UI represents the number of double bonds per 100 acyl chains and is calculated as UI = (% monoenoics) + (2*% dienoics) + (3* % trienoics) + (4*% tetraenoics)+ (5*% pentaenoics) + (6*% hexaenoics). PI indicates the susceptibility of fatty acids to peroxidative damage and is calculated with the following formula: PI = (0.025*% monoenoics) + (1*% dienoics) + (2*% trienoics) + (4*% tetraenoics) + (6*% pentaenoics) + (8*% hexaenoics). Darveau *et al.* (2005) showed that the relationships between body mass and various physiological variables in Euglossine orchid bees are expressed as power functions, where $Y=aX^b$. Moreover, relationships between body mass and FA relative abundance in birds and mammals also follow power functions (Hulbert *et al.*, 2007). Therefore, variables were log-transformed to linearize the data. Normality of residuals was also verified using the Shapiro-Wilk test.

We used linear regressions to test for relationships between the relative fatty acid abundance in each fraction and body mass. Values were weighted as a function of the sample size (N). An ANCOVA test was used to evaluate the effect of the genus and body mass on the relative abundance of fatty acids. Kolmogorov-Smirnov and Levene tests were used to test for normality and homoscedasticity of the data.

To assess and correct for the degree of relatedness between species, we analyzed the data using phylogenetic comparative methods in two steps. We first evaluated the presence and strength of a phylogenetic signal in the data. Secondly, we conducted an analysis of phylogenetically independent contrasts. Both analyses were done using the phylogenetic tree (Figure 2.2) obtained from RNA polymerase II (*RNAP II*), cytochrome oxidase subunit 1 (*CO1*), arginine kinase (*argK*), and elongation factor 1-alpha (*EF-1 alpha*) gene sequences. To generate the tree, we first compiled the sequences for our 22 species using GenBank based on the analysis found in Ramírez *et al.* (2010). We included two out-group species in the analysis (*Apis cercana* and *Apis dorsata*), as well as *Aglae caerulea*, the putative ancestral genus at the base of the orchid bees tribe. We then aligned the sequences obtained using the MUSCLE software (Edgar, 2004), and we determined the best fit model using the MrModel software (Nylander, 2004). The aligned sequences were concatenated into a singular long sequence with the help of a Perl script, putting a "?" in place of the missing data. We used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) to reconstruct the phylogeny through a partitioned dataset (4 independent genes) and an averaged Generalized Time Reversible (GTR) gamma model was used. Through an MCMC method 6 million trees were generated, keeping every thousandth generation, giving a total of 6000 trees. To measure the phylogenetic signal, we used the *phylosig* function (v0.2) included in the *phytools* package (Revell, 2012), in the R environment (R Development Core Team, 2012). We then conducted an analysis of phylogenetically independent contrasts (PIC) using the PDAP (Midford et al., 2003) module in Mesquite (Maddison and Maddison, 2004). We obtained standardized independent contrasts from the log-transformed character data. To further assess the influence of the phylogeny topology and branch length on the PIC results, we conducted all analyses using the last 2041 trees obtained from the Bayesian analysis; the same analysis was performed on a set of 10 000 simulated trees and yielded essentially the same results (not shown).

Results

The thoracic membranes of sampled orchid bee species were composed of five major fatty acids, where 16:0 and 18:0 were the main saturated fatty acids (SFA), 18:1 (n-9) was the main monounsaturated fatty acid (MUFA), 18:2(n-6) and 18:3(n-3) were the main polyunsaturated fatty acids (PUFA). Other fatty acids were found in relatively low abundances (<1%) and their presence varied among individuals of a species: 14:0, 16:1, 20:0, 20:1, 21:0, 22:0, 22:1, 20:5, 24:0 and 22:6. Mean fatty acid abundances in the PL fraction for the 4 genera studied are presented in Table 2.2.

Fatty acids in the phospholipid fraction of the thorax in Euglossine orchid bees.

The relative abundance of three fatty acids in the phospholipid fraction changed significantly with body mass. The strongest relationship found was the increase in oleic acid proportion (18:1) with increasing body mass (r^2=0.57, P<0.001) presented in Figure 2.3. This fatty acid had the highest abundance in the membrane, ranging from 43.4% in *Euglossa sapphirina* (smallest species) to 59.8% in *Eulaema bombiformis* (second-biggest species). An ANCOVA including genus as a covariate showed an interaction between body mass and genus (Mass: $F_{(2,175)}$=100.5, P<0.001; Genus: $F_{(2,175)}$=14.6, P<0.001; Genus*Mass: $F_{(2,175)}$=21.3, P<0.001). Further analysis on genera showed positive relationships for *Eufriesea* (r^2=0.85, P<0.001), *Euglossa* (r^2=0.08, P=0.004) and *Eulaema* (r^2=0.62, P<0.001), where within each genus larger species have a higher 18:1 abundance in their membranes. Pairwise comparisons showed significant differences in slopes between *Euglossa* and *Eufriesea* (P<0.001), *Eufriesea* and *Eulaema* (P<0.001), but no difference in slope (P=0.365) or intercept (P=0.065) between *Euglossa* and *Eulaema*.

Figure 2.4 shows the abundance of the two main polyunsaturated fatty acids, and total PUFA, as a function of body mass. Linoleic acid, 18:2(n-6) did not vary significantly with the orchid bees' mass (r^2=0.12, P=0.121; Fig. 2.4A). This lack of an overall significant trend was due to different relationships across genera shown by the ANCOVA (Mass: $F_{(2,175)}$=8.1 , P=0.005; Genus: $F_{(2,175)}$=270.9, P<0.001; Genus*Mass: $F_{(2,175)}$=274.7, P<0.001). Regressions within genera showed an important increase in %18:2 with mass among *Euglossa* species (r^2=0.85, P<0.001), whereas the opposite relationship could be observed among *Eufriesea* species (r^2=0.89, P<0.001). *Eulaema* species did not vary significantly with mass (r^2=0.02, P=0.414). These opposing relationships between genera explained the overall "bell-shaped" curve for this polyunsaturated fatty acid.

Alpha-linolenic acid, 18:3(n-3) decreased significantly (r^2=0.29, P=0.009) with body mass, as portrayed in Figure 2.4B. The abundance varied from 25.7% in the smallest species (*Euglossa sapphirina*) to 5.4% (*Eufriesea dressleri*). An ANCOVA showed a significant interaction between body mass and genus acting on 18:3 abundance (Mass: $F_{(2,175)}$=0.873, P=0.351; Genus: $F_{(2,175)}$=30.0, P<0.001; Genus*Mass: $F_{(2,175)}$=36.3, P<0.001). The strongest decrease in 18:3 abundance was found within the genus *Euglossa* (r^2=0.50, P <0.001) while *Eufriesea* species showed a slight increase in 18:3 (r^2=0.14, P=0.023). As with linoleic acid, there was no clear trend in the genus *Eulaema* (r^2=0.03, P=0.280).

The abundances of linoleic, linolenic and other trace polyunsaturated fatty acids were combined to obtain the total %PUFA (Fig. 2.4C). There was no significant change in the relative abundance of PUFAs as a function of body mass (r^2=0.15, P=0.072). Abundance values ranged from 17.5% in *Eufriesea dressleri* (6th biggest species) to 32.4% in *Euglossa imperialis* (medium-sized species), an almost two-fold variation in PUFA content. Results from the ANCOVA revealed a significant interaction between body mass and genus (Mass: $F_{(2,175)}$=59.1, P<0.001; Genus: $F_{(2,175)}$=30.7, P<0.001; Genus*Mass: $F_{(2,175)}$=25.9, P<0.001). Since 18:2 and 18:3 were the most abundant PUFAs, the observed trends for the different genera reflect the sum of these fatty acids. The genus *Euglossa* had strong opposing relationships in linoleic and linolenic acids (increasing and decreasing with mass, respectively). This resulted in a lack of variation of total PUFAs with body mass (r^2=0.003; P=0.578). *Eufriesea* showed a slight decrease in 18:3 with mass, while the abundance of 18:2 decreased markedly with body mass. When combined, this resulted in a decrease of PUFAs with increasing body mass (r^2=0.54, P<0.001). As for the genera *Eulaema*, despite the lack of trend in the two important PUFAs, the total abundance of polyunsaturates decreased with body mass (r^2=0.41, P<0.001).

The relative abundance of palmitic, stearic and the sum of saturated fatty acids were plotted against body mass in Figure 2.5. %16:0 did not vary with body mass in orchid bees (r^2=0.05, P=0.338; Fig. 2.5A). This fatty acid was also the less abundant in the membrane, ranging from 4.4% (*Eufriesea pulchra*) to 13% (*Eufriesea dressleri*). An ANCOVA showed a significant interaction of body mass and genus (Mass: $F_{(2,175)}$=2.2, P=0.142; Genus: $F_{(2,175)}$=5.9, P=0.003; Genus*Mass: $F_{(2,175)}$=4.9, P=0.009). There was a slight decrease in palmitic acid abundance among *Eulaema* species (r^2=0.44, P<0.001), while abundance increased in *Euglossa* (r^2=0.07, P=0.005) and did not vary in *Eufriesea* (r^2=0.005, P=0.681). There were differences in mean values between *Euglossa* and *Eufriesea* (P=0.001), but not *Eufriesea* and *Eulaema* (P=0.105). Stearic acid (18:0) abundance, on the other hand, varied importantly among Euglossine orchid bees. %18:0 decreased significantly (r^2=0.35, P=0.004; Fig. 2.5B) with body mass. This saturated fatty acid was found in slightly higher abundance than palmitic acid in the membrane, ranging from 8.9% (*Eulaema cingulata*) to 18.2% (*Eufriesea dressleri*). There was a significant interaction between body mass and genus (Mass: $F_{(2,175)}$=26.7, P<0.001; Genus: $F_{(2,175)}$=11.8, P<0.001; Genus*Mass: $F_{(2,175)}$=4.1, P=0.019). Among *Euglossa* species, the relationship was stronger (r^2=0.46, P<0.001) than among *Eufriesea* (r^2=0.10, P=0.048). There was no relationship in *Eulaema* species (r^2=0.02, P=0.422). The abundances of palmitic, stearic and other trace saturated fatty acids were combined into the total SFA abundance presented in Figure 2.5C. There was a significant decrease in total saturated fatty acid with body mass (r^2=0.30, P=0.008). There was no significant interaction of body mass and genus (Mass: $F_{(2,175)}$=11.5, P=0.001; Genus: $F_{(2,175)}$=4.6, P=0.011; Genus*Mass: $F_{(2,175)}$=0.449, P=0.639). Relative abundance of SFA varied from 15.7% (*Eulaema cingulata*) to 32.6% (*Eufriesea dressleri*). Among genera, there was a significant decrease in SFA in *Euglossa* species (r^2=0.10, P=0.001) but not in *Eufriesea* (r^2=0.04, P=0.199) nor in *Eulaema* (r^2=0.08, P=0.076).

Unsaturation index, the number of double bonds per 100 acyl chains, did not vary significantly with body mass (r^2=0.16, P=0.07; Fig. 2.6A). Results from the ANCOVA revealed no significant interaction between body mass and genus (Mass: $F_{(2,175)}$=12.3, P=0.001; Genus: $F_{(2,175)}$=8.4, P<0.001; Genus*Mass: $F_{(2,175)}$=2.3, P=0.1). Among genera, there was no significant variation in *Eufriesea* (r^2=0.051, P=0.172) and *Eulaema* (r^2=0.024, P=0.357), but bigger *Euglossa* had significantly less double bonds (r^2=0.21, P<0.001). Post-hoc tests revealed no significant differences in slopes between each genera, but there were differences in mean values between *Euglossa* and *Eulaema* (P=0.002) and between *Eufriesea* and *Eulaema* (P<0.001), but not between *Euglossa* and *Eufriesea* (P=0.99). The sensitivity of phospholipids to peroxidative damage, peroxidation index, decreased significantly with mass (r^2=0.36; P=0.003; Fig. 2.6B) in Euglossine bees. There was a significant interaction between body mass and genus (Mass: $F_{(2,175)}$=33.9, P<0.001; Genus: $F_{(2,175)}$=9.9, P<0.001; Genus*Mass: $F_{(2,175)}$=5.8, P=0.004). The decrease with mass was found among all the genera investigated: there were significant decreases in PI in *Eufriesea* (r^2=0.22, P=0.003), *Euglossa* (r^2=0.22, P<0.001) and *Eulaema* (r^2=0.27, P=0.001). Post-hoc tests showed significant differences in slopes between *Euglossa* and *Eulaema* (P=0.007), *Eufriesea* and

Eulaema (*P*=0.004), but not between *Euglossa* and *Eufriesea* (*P*=0.077), between which there were no differences in mean values (*P*=0.127).

Fatty acid composition of the membrane and the influence of phylogeny

The presence of a phylogenetic signal, the tendency of related species to resemble each other, was assessed and quantified for the fatty acids found in the PL fraction. In two fatty acids, phylogenetic signal was detected, both using Pagel's λ and Blomberg's *K* indices. 18:0 had a significant phylogenetic signal (λ=1, *P*<0.001 and *K*=0.92, *P*=0.001). 18:1 also had a significant signal (λ=0.98, *P*=0.03 and *K*=0.69, *P*=0.02). The total SFA had a significant signal only when calculated using Pagel's λ index (λ=0.74, *P*=0.04) but not when looking at Blomberg's *K* (*K*=0.51, *P*=0.11). No significant signal was detected for 16:0 (λ=0, *P*=1 and *K*=0.29, *P*=0.83), 18:2 (λ=0, *P*=1 and *K*=0.39, *P*=0.4), 18:3 (λ=0.16, *P*=0.51 and *K*=0.41, *P*=0.3), PUFA (λ=1.04, *P*=0.26 and *K*=0.42, *P*=0.28), UI (λ=0.18, *P*=0.59 and *K*=0.38, *P*=0.4) and PI (λ=0.22, *P*=0.62 and *K*=0.45, *P*=0.17).

The relationship between body mass and fatty acid composition of the membrane was corrected for phylogeny in the fatty acids where a phylogenetic signal was detected. The values for gradual and speciational evolution were not different for the fatty acids analyzed (not shown). PIC analysis showed that the body mass effect on %18:1 remained significant when correcting for phylogenetic relationships (r^2=0.3, *P*=0.004). The average correlation coefficient of the distribution (*r*) was 0.55, with a critical *r* value of 0.3598 (one-tailed test; d.f.=20; Fig. 2.7A and B). Similarly, the relationship between body mass and %18:0 remained significant when applying the PIC analysis (r^2=0.15, *P*=0.03; Fig. 2.7C), and the correlation coefficient *r* was -0.39, which slightly reduced the number of trees for which *r* remained significant (1898 out of 2041 trees; Fig. 2.7D). On the other hand, the relationship between total SFA and body mass did not stay significant when correcting for phylogeny (r^2=0.03, P=0.21; Fig. 2.7E) and the critical value for which r was significant was higher than the resulting r value of -0.18 (Fig. 2.7F). The PIC analysis results obtained from the set of 10 000 simulated tree were globally the same for 18:1, 18:0 and total SFA.

Neutral lipid and non-esterified fatty acids of the thorax in Euglossine orchid bees.

Relative abundances of fatty acids in the neutral lipid and non-esterified fatty acids fractions of the thorax were analyzed. Table 2.3 reports the results for these two fractions and the major fatty acids (abundances >5%) that were found in them.

The NL fraction contained more long-chain polyunsaturated fatty acids compared to other, but their abundances were very low compared to the five main fatty acids found in the PL fraction. %16:0 did not vary with body mass (r^2=0.13, P =0.572), while %18:0 decreased significantly with mass (r^2=0.76, P<0.001) and the total %SFA followed the same trend (r^2=0.68, P<0.001). Moreover, %18:1 was the most abundant fatty acid in this fraction and increased significantly with increasing body mass (r^2=0.84, P<0.001). These trends were identical to those found in the PL fraction (see previous section). However, the trend for %18:2 was different in the NL fraction. %18:2 decreased significantly with body mass (r^2=0.24, P<0.001), while %18:3 decreased with mass (r^2=0.49, P<0.001), just as in the PL. When all the long-chain polyunsaturated fatty acids were combined (including the trace fatty acids) there was an overall significant decrease in %PUFA (r^2=0.48, P<0.001), which differed from the result found in PL. The number of double-bonds per 100 acyl chains, unsaturation index, slightly increased with body mass in NL (r^2=0.04, P=0.008), contrary to

PL. Peroxidation index however, decreased significantly with mass like in the phospholipid fraction (r^2=0.45, P<0.001), and double-bond index increased slightly (r^2=0.41, P<0.001).

Abundance results from the NEFA fraction had minor differences with abundances in the PL fraction, specifically in terms of PUFA abundance. However, the trends were similar to those found in the PL for the other classes of fatty acids. In the saturated fatty acids, %16:0 decreased slightly with mass (r^2=0.11, P<0.001), and %18:0 decreased significantly (r^2=0.27, P=0.03). Overall, the sum of SFA also decreased significantly with mass (r^2=0.25, P=0.087). The MUFA 18:1 increased significantly with mass, a result found consistently in all three fractions (r^2=0.58, P<0.001). In the PUFAs, %18:2 had a slight increase with mass (r^2=0.1; P<0.001), while %18:3 decreased with mass (r^2=0.12; P<0.001). Consequently, the sum of PUFAs stayed constant across body masses (r^2=0.001; P=0.655), a result only found in the NEFA fraction. Like in the NL, there was a slight increase in the unsaturation index (r^2=0.08, P<0.001) and in double-bond index (r^2=0.13, P<0.001). Contrary to the other two fractions, however, peroxidation index did not vary with mass in the NEFAs (r^2=0.004, P=0.415). Results from the ANCOVAs on mass and genus for both NL and NEFA fractions are listed in Table 2.4.

Discussion

The scaling of membrane composition with body mass in orchid bees

The main finding of this study is that the membrane lipid composition of tropical orchid bee flight muscles is composed of five main fatty acids, and their abundances vary with body mass. The significant increase of oleic acid (18:1; Fig. 2.3) and the decreases of alpha-linolenic (18:3; Fig. 2.4B) and stearic acid (18:0; Fig. 2.5B) with body mass provide support for the idea of a role for fatty acids in setting the pace of life, the "membrane pacemaker" theory of metabolism developed by Hulbert and Else (1999). As predicted by this theory, the principal MUFA increases with mass in Euglossine bees, just as it does in mammals and birds (Hulbert, 2003). In addition, these results are globally consistent across the three lipid fractions (PL, NL and NEFA) analyzed in the thorax. The abundance of 18:1 in mammalian and bird membranes spans roughly 5 to 30% from mice to cattle or from hummingbirds to emus. In our invertebrate model, its abundance varies roughly between 40 and 60%, and it is the largely dominant fatty acid. This major difference between vertebrates and invertebrates could have important functional implications that will be discussed further on. To our knowledge, this is the first evidence of a variation of MUFA with mass among species of a phylum different from chordates, and with such a narrow range in body mass (22-fold range versus 9 000 and 11 000-fold range in mammals and birds, respectively).

Contrary to our predictions, total PUFA content did not decrease significantly with body mass (Fig. 2.4C). The reason for this lack of significance lies in the fact that there were different trends across genera for one of the two important PUFAs, 18:2(n-6) (Fig. 2.4A). The relative abundance of this fatty acid did not vary significantly overall for Euglossine bees, because its abundance increased among smaller species, whereas it decreased in bigger bees. The other main PUFA, 18:3(n-3) followed the predictions of the "membrane pacemaker" theory, as its relative abundance decreased significantly with mass (Fig. 2.4B). In mammals and birds, an omega-3 fatty acid, 22:6 (docosahexaenoic acid, DHA) decreased with size (Hulbert, 2003). Here, we showed that another omega-3 fatty acid, alpha-linolenic acid (18:3, n-3) partially compensated for the increase in 18:1 oleic acid among Euglossine bees. This feature common to both groups and bees is perhaps not surprising. Dietary intake of omega-3 fatty acids (specifically, DHA) has been shown to be linked to increases in metabolic rates; they also have been proposed to be implicated in lifespan and in various metabolic diseases (Hulbert *et al.*, 2005; Valencak and Ruf, 2007). Moreover, a novel finding is the important contribution of stearic acid (18:0), a SFA. This is the first evidence of a systematic decrease of a saturated fatty acid with mass, as there is no mention of any variation of SFA with mass in previous studies. This could be linked to the fact that SFAs in animals are converted to UFA by desaturase complexes bound to the membrane (Hulbert and Else, 1999). Stearic acid followed the trend predicted for PUFAs, and its relative abundance in the membrane was similar to that of unsaturates. Therefore, we cannot overlook a potential role for this class of fatty acids in setting the "pace" of reactions.

Contrary to what Hulbert and colleagues found in mammals and bird tissues, the overall unsaturation index (or the number of double bonds per 100 acyl chains) did not decrease significantly with mass (Fig. 2.6A). This is presumably due to the important weight of 18:1 MUFA in the calculation as a consequence of its very high abundance in Euglossine bees as opposed to vertebrates, as discussed above. The presence of a significant negative trend in the genus *Euglossa* shows that differences among genera might be important. Therefore, specific differences between genera add another level of complexity to the membrane composition–body mass relationships.

It has been previously established that metabolic rate during hovering flight scales allometrically with body mass in carpenter bees (Chappell, 1982) and in orchid bees (Casey *et al.*, 1985). This influence of mass in the latter species was found to be mediated by wing morphology and wingbeat frequency, which was shown to be linked to mass-specific metabolic rate even after controlling for phylogenetic relatedness and body mass variation

(Darveau *et al.*, 2005a). Furthermore, a subsequent study found that various muscle enzymes involved in substrate catabolism scale allometrically with body mass, although Hexokinase is the only one that was significant when controlling for phylogenetic relatedness (Darveau *et al.*, 2005b). These studies provided a framework for the interpretation of body mass effects on physiology and metabolism, and suggested that integrative analysis on scaling of metabolism should combine mechanistic and evolutionary approaches at the micro- and macro-physiological levels. Here, we have brought new elements to further strengthen this framework by showing that membrane composition in orchid bees also scales with body mass. Moreover, we have used phylogenetic approaches to show that these relationships hold true when controlling for evolutionary distance among species.

Membrane composition and the influence of phylogeny

Previous studies done on the "membrane pacemaker" theory of metabolism generally overlooked the problem of phylogenetic relatedness among the species studied. This issue of a lack of independence in the data when comparing various traits was raised early on in comparative studies (Felsenstein, 1985), and more recently revisited in the context of membrane composition and aging by Speakman (2005). His reanalysis of relationships between DHA content and maximum lifespan in 8 mammalian species incorporating the phylogenetically independent contrasts method showed that there was no significant relationship between the abundance of this important PUFA and longevity, contrary to what was previously shown. The author insisted on the necessity of accounting for differences in body mass between species, and the phylogenetic dependence of comparative data, two common problems in comparative analyses. A subsequent study with a more important dataset of 42 mammalian species by Valencak and Ruf (2007) showed no relationship between membrane composition and basal metabolic rate or maximum lifespan when correcting for phylogenetic relatedness. The important issue of relatedness among species studied for membrane composition-body mass relationships, however, has been overlooked in Hulbert's original studies.

The presence of a phylogenetic signal for 18:1 in our study indicates an influence of relatedness among species on oleic acid content. However, when correcting for phylogenetic relationships, the increase in 18:1 with body mass remained significant (Fig.2.7A). Moreover, 18:0, the other fatty acid where a phylogenetic signal could be detected, also had a significant negative relationship with body mass when correcting for phylogeny (Fig. 2.7C). A significant signal was also detected in SFA (but only when using Pagel's λ index), but the relationship between these fatty acids and body mass disappeared when corrected for phylogeny (Fig. 2.7 D). Thus, body mass seems to be the most important factor driving variation in 18:0 and 18:1, but not in total SFA (presumably due to the lack of signal in 16:0). In general, the results obtained suggest a very fine regulation of saturated and monounsaturated 18-carbon fatty acids across genera and mass, and a high variability at the polyunsaturated fatty acid level. The former being the dominant type of fatty acids in the membrane, it is perhaps logical that variations in its abundance could exert a bigger influence on the overall physiology of the membrane. For instance, in a phospholipid the pair of fatty acids consist typically of a saturated fatty acid associated to an unsaturated fatty acid (Hulbert and Else, 1999). Here, a different portrait of the organisation of the membranous phospholipids could exist, as a monounsaturated fatty acid already forms more than 40% of the total abundance, much more *per se* than any other class of fatty acids. Thus, contrary to mammals and birds where long-chain PUFAs are highly abundant, in bees there seems to be interplay between three types of 18-carbon fatty acids, mainly a MUFA, a SFA and two PUFAs. The systematic changes in fatty acid composition with mass, somewhat independent of phylogeny, revive the interest in the validity of the "membrane pacemaker" theory of metabolism.

Globally, the variation in fatty acid composition in orchid bees seems to be constant across genera for saturated and monounsaturated fatty acids, but not for polyunsaturated fatty acids where there are important inter-genera differences. The mid-sized species (0.21 to 0.5g in body mass) of the genus *Eufriesea* (in red in all figures) followed more closely the trends predicted by the "pacemaker" theory for each fatty acid. They showed no change in SFA abundance (a marginal one in 18:0) and a predicted increase in 18:1. Contrary to the other genera, 18:2 proportion decreased significantly with size in this genus, while 18:3 increased slightly, resulting in an important decrease in total PUFAs. The smaller sized species of the genus *Euglossa*, presented more contrasted results. As predicted and constant across all genera was the positive increase in 18:1 abundance, while the abundance increased slightly for 16:0 and decreased for 18:0, globally decreasing for total SFAs, contrary to our predictions. The PUFA 18:3 was found to decrease significantly in *Euglossa*, as predicted. The key aspect that distinguishes *Euglossa* from the other genera and goes against the predictions of the "pacemaker" theory is the important increase in 18:2 abundance with mass. This increase is the mirror image of the decrease found in *Eufriesea* for this fatty acid, and is a novel aspect brought by our study. Moreover, the abundance of a particular fatty acid in the thorax seems to be a well conserved trait for each species, as illustrated by the small error bars. Thus, the question of what sets the membrane composition for a particular species or a set of species inside a genus is of particular interest. It has been previously established that diet and development have strong influences on fatty acid profiles in various insect species (Stanley-Samuelson *et al.*, 1988). The important differences found across different genera of Euglossine bees could therefore be due to differences in diet during development of the larvae, to genetic differences in membrane lipogenic enzymes, or both.

While fatty acid composition at the inter- and intra-specific levels of Euglossine bees has been clarified and the possible role of ecological and genetic factors have been suggested, the implications at the functional level need to be scrutinized and put back in the context of the “membrane pacemaker” theory of metabolism.

Functional implications of changes in membrane composition

According to the “membrane pacemaker” theory of metabolism, fatty acid composition of the membrane is the evolutionary modulator of cellular metabolic rate of different species (Hulbert, 2007). The theory puts emphasis on the potential role of DHA content, an abundant polyunsaturated fatty acid in the membrane of small vertebrates (Hulbert, 2003). The fact that DHA modulates the level of membrane proton leakage and the activity of sodium pumps is well established (Haggerty *et al.*, 2008) and argued as a potential mechanistic explanation for changes of overall metabolic rate among organisms. In our study, oleic acid is the most abundant fatty acid and the most variable among species of different sizes. Similarly to DHA, variation in oleic acid content has been shown to induce changes in membrane properties such as fluidity, permeability, protein activities and proton conductivity (Brunaldi *et al.*, 2005). This is presumably due to the molecular shape of this fatty acid and its effect on the order-disorder membranous phase transition (Funari *et al.*, 2003). Thus, the almost 1.5-fold variation in percentage contribution of 18:1 in orchid bee thoracic membranes could imply important structural and functional effects. Whether or not these systematic changes in composition are also linked to whole-organism metabolic rates needs to be scrutinized.

A corollary of the "membrane pacemaker" theory of metabolism is the idea that fatty acid composition and maximum lifespan are linked (Pamplona *et al.*, 2000; Hulbert *et al.*, 2007). The reasoning behind this lies in the action of oxygen radicals that are soluble in the lipid membrane bilayer, and in the fact that different fatty acid chains will have different levels of susceptibility to damage. The carbons between the double-bonded carbons of the acyl chain are known to be the most susceptible to radical attacks, thus PUFA are the most damaged fatty acids, while SFA and MUFA are resistant to peroxidation. In mammals and birds, the maximum lifespan (or longevity) and the peroxidation index (PI) of the skeletal muscle are thus inversely correlated (Hulbert *et al.*, 2007). In a study on queen and worker honeybees, Haddad and colleagues (2007) found that long-lived queens (2 to 5 years) have lower PI than short-lived workers (75 to 135 days), possibly due to their diet during development. In our study, we found a decrease in the PI with body mass due to major differences in 18:1 content and much lower PUFA abundance in the bigger Euglossine species (Fig. 2.6B). However, the PI in the orchid bees' thorax is generally higher (index values between 24 and 60) than in the thorax of queen and worker honeybees (PI ranging from 5 to 35). In fact, the values we have found in orchid bees for PI are closer to those found in the head and abdomen of worker honeybees (index values between 15 and 70).

These differences are largely due to 18:3 (n-3), for which the mean abundance is 2 to 3 times higher in Euglossine bees than in the thorax of workers and queen honey bees, but is comparable to the abundance found in their heads (a consequence of the pollen diet). The study on honeybees suggests a link between longevity and different castes among highly eusocial insects. Little is known on longevity in Euglossine bees, but there seems to be considerable variation among species, ranging from 2 to 6 months approximately (Dressler, 1982). Moreover, most orchid bees species are solitary or communal, although the number of studies is limited and there are some rare cases of weakly eusocial species (Roubik and Hanson, 2004). Longevity in our orchid species is therefore variable but closer to that of the worker rather than the queen honeybee, which concurs with relatively close peroxidation index values.

The lipid fraction is a basic constituent, along with carbohydrates and protein, of the floral pollen collected by bees (Szczesna, 2006). Lipids are also present in the nectars of flowers consumed by Hymenopterans (Kevan and Baker, 1983). Many insects require an adequate lipid content in their diet for a proper metamorphosis (Canavoso *et al.*, 2001), and the absence of essential dietary fatty acids has been shown to cause abortion at the adult moult or dysfunction at the adult stage (Stanley-Samuelson *et al.*, 1985). In honeybees, a low or high-lipid loaded diet can have negative consequences on the longevity of the bee (Manning *et al.*, 2007). Pollen and nectar diet with various fatty acid profiles may affect the orchid bees' membrane composition. Orchid bees do not only visit orchid flowers (which are typically specific to one or two male species), but gather their resources from a variety of plants. Some plants are visited by a limited amount of species and by female or male Euglossine bees only (Bawa, 1990). In fact, female orchid bees visit an estimated 75 plant species from 45 different families for pollen, and both males and females visit 121 species from 25 different families for nectar (Ramírez *et al.*, 2002). Maize (*Zea mays*) and palm species of the family *Arecaceae* are examples of plants visited by orchid bees for their pollen (Roubik and Hanson, 2004). Manning (2006) showed that the pollen of various palm species and of maize was very rich in 18:2 and 18:3 respectively, with abundance values of 40% of the total lipid content of the pollen. Other examples of plants rich in PUFAs include those of the family *Asteraceae*, in which flowers from at least two genera are visited by orchid bees; therefore it is possible that bees can obtain these PUFA from their nectar and pollen. Extensive and detailed records of the plants visited by each species of orchid bees could not be found, along with their respective pollen and nectar lipid composition. Such records could reveal possible links between the bees' diet and their membrane composition, and assess the relative role of diet versus biosynthesis of fatty acid in the membranes, since all insects do not seem to have the ability to biosynthesize 18:2 (n-6) and 18:3 (n-3) (Stanley-Samuelson *et al.*, 1988). For now, we can only suggest that the species groupings found for this PUFA emerge in part due to pollination and diet.

Conclusions

This study has shown that, in agreement with the "membrane pacemaker" theory of metabolism, the phospholipid composition of the thorax varies systematically with body mass in various species of orchid bees. We have found that smaller species of Euglossine bees have lower amounts of a MUFA, 18:1 (n-9), than bigger species. This increase in 18:1 resembles the one observed in mammals and birds, although oleic acid is the most abundant fatty acid in our model, contrary to the two vertebrate models where its abundance was lower. This is the first evidence of a systematic variation with mass in invertebrates and at a narrow range in body mass. Moreover, these systematic variations were also present in the NL and NEFA fractions of thoracic lipids. Contrary to our predictions, however, total PUFA content did not vary systematically with body mass, due to major differences in the variation of 18:2 (n-6) and 18:3 (n-3), the two main PUFAs in our invertebrate model. Indeed, 18:3 (n-3) follows the predictions of the pacemaker theory, as its abundance decreases with body mass, much like DHA in mammals and birds. On the other hand, the trends for 18:2 differ across genera and it is hypothesized that these differences could be linked to the ecology and diet of the different species. A novel result in the context of membrane composition-body mass studies is that in Euglossine bees, the SFA 18:0 (stearic acid) decreases systematically with body mass. Stearic acid being as abundant as 18:3, the combination of these two fatty acids could compensate for the important increase in 18:1. Unsaturation index, or the number of double bonds per 100 acyl chains, did not vary significantly with mass, contrary to the results obtained by Hulbert (2003). This is presumably due to the important abundance of 18:1. These results offer a new perspective on the "membrane pacemaker" theory of metabolism and provide new information for the interpretation of body mass effects on physiology and metabolism.

While previous studies on membrane composition-body mass relationships generally overlooked the importance of phylogenetic relatedness among species, our study has integrated this aspect using statistical methods. In two fatty acids where the presence of a phylogenetic signal could be detected, 18:1 and 18:0, we have found that the relationships with body mass were maintained when correcting for phylogeny. Moreover, the important differences in trends among genera for the PUFA 18:2 (linoleic acid), and the observed decrease in peroxidation index with mass suggested potential functional implications for membrane composition in Euglossine bees. Indeed, orchid bees have a peroxidation index and longevity similar to that of worker honeybees, thus membrane composition could play a role in determining lifespan. Pollen and nectar lipidic composition of various plants visited by orchid bees suggest that the observed groupings for linoleic acid abundance could be due to differences in species' diets.

To conclude, our results revive the interest for the "membrane pacemaker" theory of metabolism proposed by Hulbert and Else. The differences with the original theory suggest a more complex model where not only PUFAs and MUFAs could play a role in "setting the pace" for metabolism, but also SFAs. Whether these changes in membrane composition with body mass have the predicted metabolic implications needs to be scrutinized by combining these results with whole-animal measurements of metabolic rate.

Figures and tables

Figure 2.1. Euglossine bees show great variation in size among species. Here, two orchid bee species analyzed in the study: *Euglossa mixta* (left) with a mean mass of 108mg (±4mg) and *Eulaema meriana* (right) with a mean mass of 939mg (±25mg). (Photo: C.-A. Darveau)

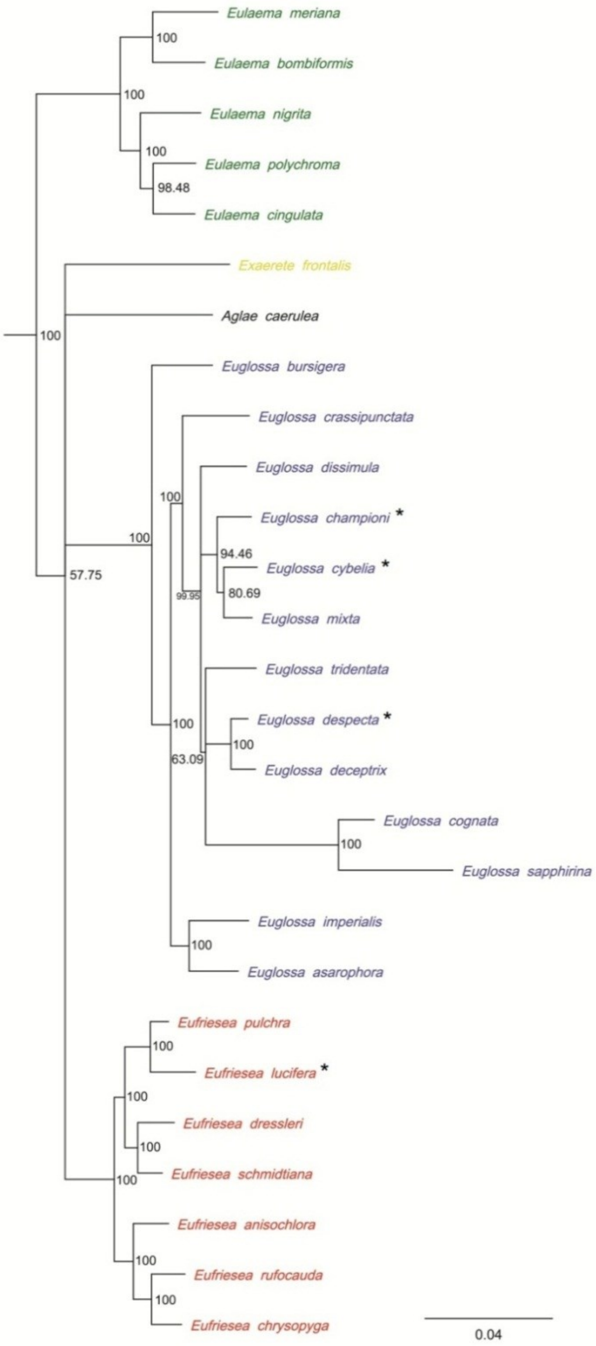

Figure 2.2. Phylogenetic tree hypothesized for 26 orchid bee species based on *RNAPII, CO1, argK* and *EF-1alpha* sequences (see Table 2.5 for accession numbers) and inferred using the Bayesian method. Posterior probability distribution values are shown at the nodes, and each of the three genera is presented in a different color, with the out-group used for the phylogeny in dark. * indicates the four species pruned from the tree for the subsequent analysis (see Table 2.1 for a list of the 22 species).

Table 2.1. Sampled orchid bee species ranked by mean body mass (g) (±SE) (*n*=188).

Genus	Species	*n*	Body mass (g)
Euglossa	*sapphirina*	12	0.060 (±0.002)
Euglossa	*crassipunctata*	14	0.076 (±0.002)
Euglossa	*bursigera*	12	0.081 (±0.002)
Euglossa	*dissimula*	10	0.092 (±0.003)
Euglossa	*deceptrix*	6	0.095 (±0.005)
Euglossa	*mixta*	10	0.108 (±0.004)
Euglossa	*tridentata*	12	0.121 (±0.004)
Euglossa	*cognata*	13	0.162 (±0.004)
Euglossa	*asarophora*	5	0.162 (±0.008)
Euglossa	*imperialis*	11	0.169 (±0.006)
Eufriesea	*rufocauda*	5	0.212 (±0.014)
Eufriesea	*anisochlora*	9	0.219 (±0.007)
Eufriesea	*chrysopyga*	7	0.274 (±0.007)
Eulaema	*polychroma*	2	0.333 (±0.022)
Eufriesea	*pulchra*	10	0.389 (±0.015)
Eulaema	*nigrita*	10	0.415 (±0.018)
Eufriesea	*dressleri*	3	0.451 (±0.021)
Eufriesea	*schmidtiana*	4	0.502 (±0.019)
Eulaema	*cingulata*	11	0.526 (±0.019)
Exaerete	*frontalis*	7	0.710 (±0.042)
Eulaema	*bombiformis*	4	0.923 (±0.036)
Eulaema	*meriana*	11	0.939 (±0.025)

Table 2.2. Mean (±SE) % fatty acid abundance in the PL fraction for the 4 genera of Euglossine bees studied.

Fatty acid	*Eufriesea*	*Euglossa*	*Eulaema*	*Exaerete**
C14:0	0.14 (±0.05)	0.13 (±0.07)	0.48 (±0.13)	0.02
C16:0	7.43 (±0.60)	7.36 (±0.45)	6.37 (±0.31)	8.85
C16:1 n-7	0.08 (±0.04)	0.91 (±0.21)	0.05 (±0.04)	0.26
C18:0	15.38 (±1.12)	13.39 (±0.76)	10.11 (±0.45)	9.75
C18:1 n-9	51.33 (±1.67)	48.56 (±1.86)	55.69 (±1.15)	52.59
C18:2 n-6	12.95 (±1.70)	11.29 (±1.50)	13.49 (±2.11)	16.18
C18:3 n-3	11.06 (±1.05)	16.73 (±1.35)	13.30 (±1.41)	11.91
C20:0	0.63 (±0.20)	0.62 (±0.17)	0.26 (±0.10)	0.06
C20:5	0.77 (±0.28)	0.53 (±0.17)	0.22 (±0.09)	0.03
C22:0	0.08 (±0.07)	0.01 (±0.01)	0.00 (±0.00)	0.35
C22:1	0.11 (±0.08)	0.47 (±0.20)	0.03 (±0.01)	0.00
∑PUFAs	24.79 (±1.55)	28.59 (±1.92)	27.01 (±1.49)	28.12
∑SFAs	23.52 (±1.45)	21.37 (±1.01)	16.74 (±0.70)	19.00

*: Genus *Exaerete* is composed of only one species, thus no SE is presented.

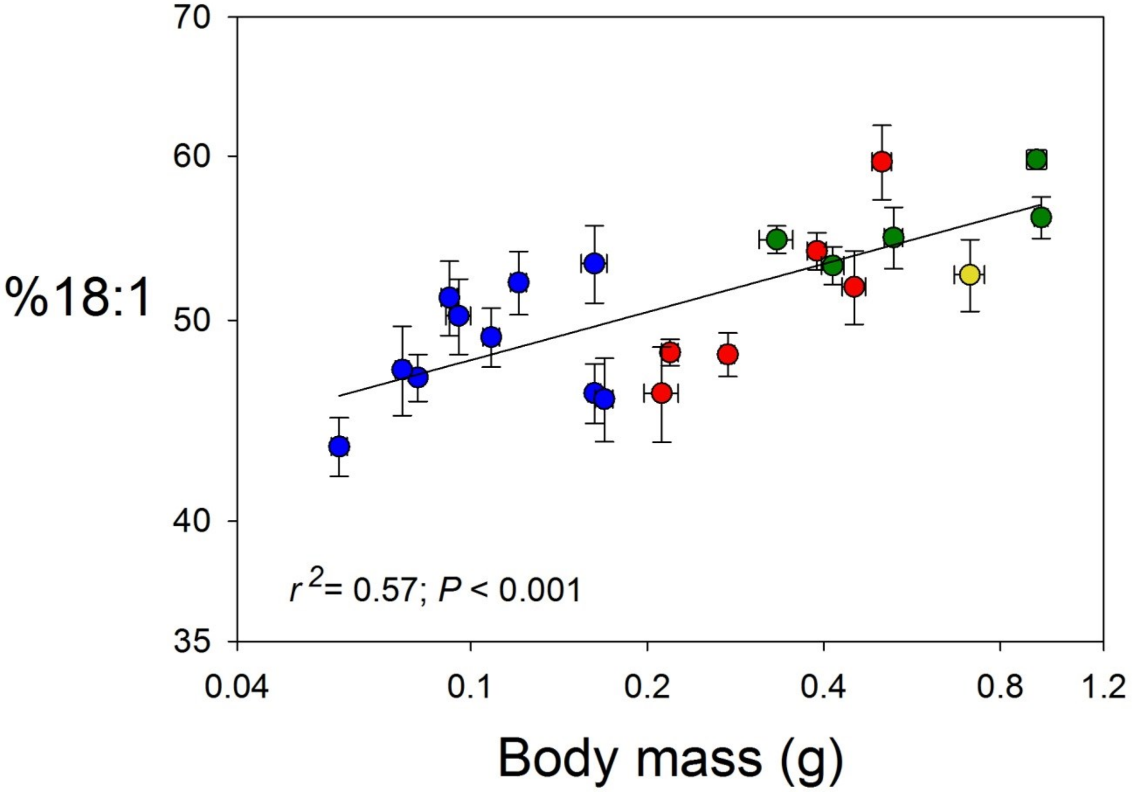

Figure 2.3. Relationship between body mass and the relative abundance of 18:1 (n-9) in the thorax membrane of 22 orchid bees species (*n*=2 to 14 individuals). Values are means (±SE). Colors represent the five genera sampled (blue: *Euglossa*, red: *Eufriesea*, green: *Eulaema*, yellow: *Exaerete*).

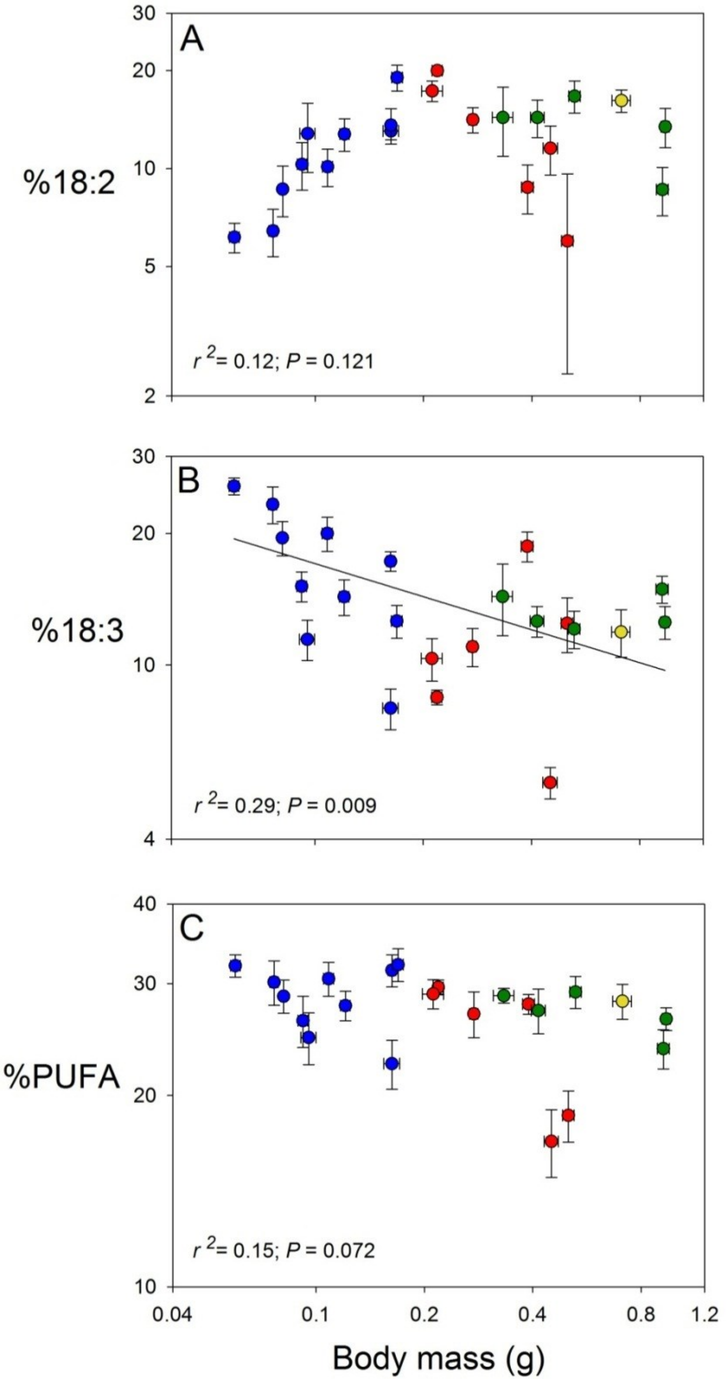

Figure 2.4. Relationship between body mass and the relative abundance of 18:2 (n-6) (panel A), 18:3 (n-3) (panel B) and total PUFAs (panel C) in the thorax membrane of 22 orchid bee species (*n*=2 to 14 individuals per species). Values are means (±SE). Colors represent the five genera sampled (blue: *Euglossa*, red: *Eufriesea*, green: *Eulaema*, yellow: *Exaerete*).

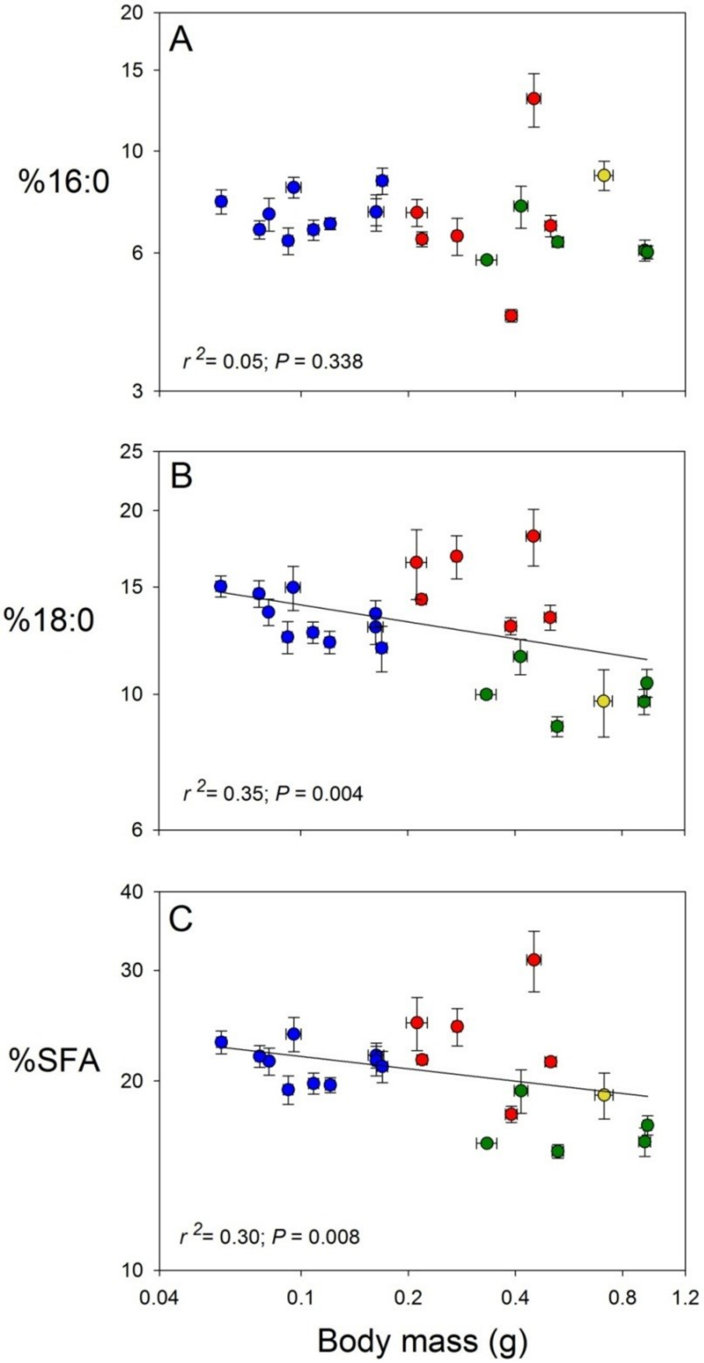

Figure 2.5. Relationship between body mass and the relative abundance of 16:0 (panel A), 18:0 (panel B) and total SFAs (panel C) in the thorax membrane of 22 orchid bee species (n=2 to 14 individuals per species). Values are means (±SE). Colors represent the five genera sampled (blue: *Euglossa*, red: *Eufriesea*, green: *Eulaema*, yellow: *Exaerete*).

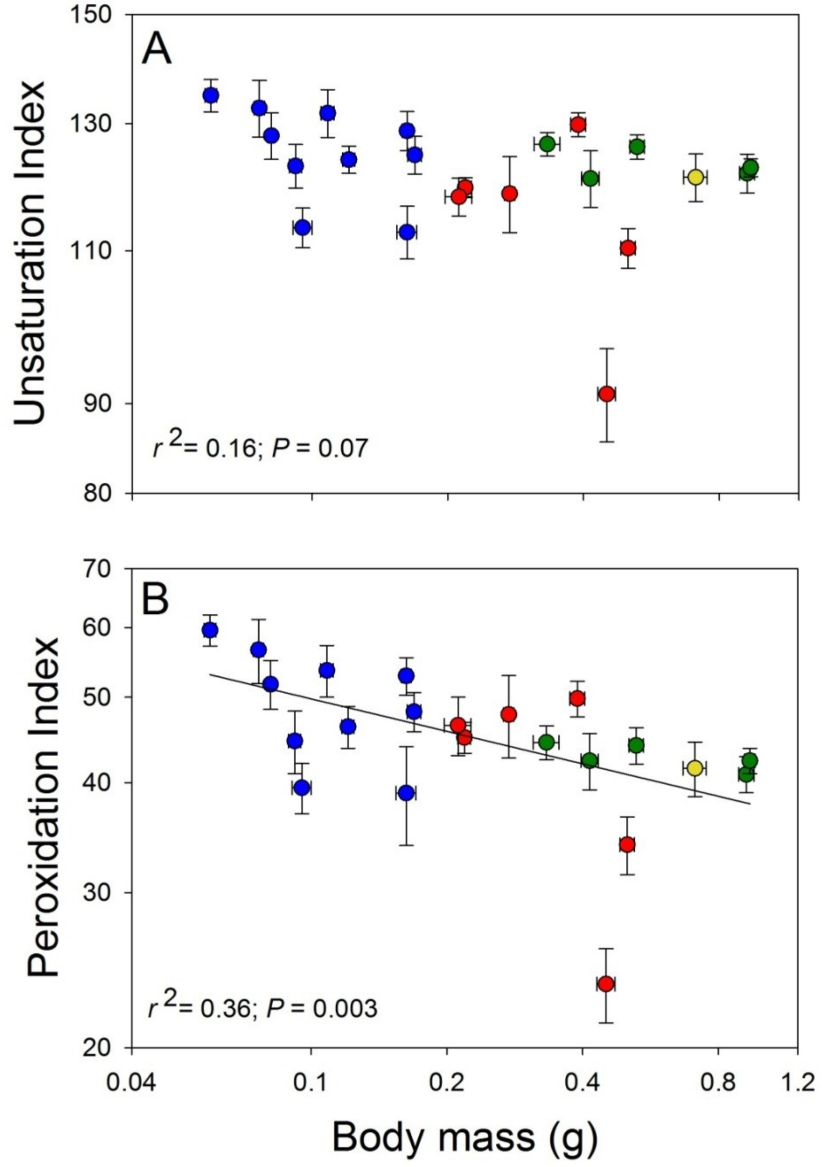

Figure 2.6. Relationship between body mass and Unsaturation index (panel A) or Peroxidation index (panel B) in the thorax membrane of 22 orchid bee species (*n*=2 to 14 individuals per species). Values are means (±SE). Colors represent the five genera sampled (blue: *Euglossa*, red: *Eufriesea*, green: *Eulaema*, yellow: *Exaerete*).

Phylogenetically independent contrasts: PL

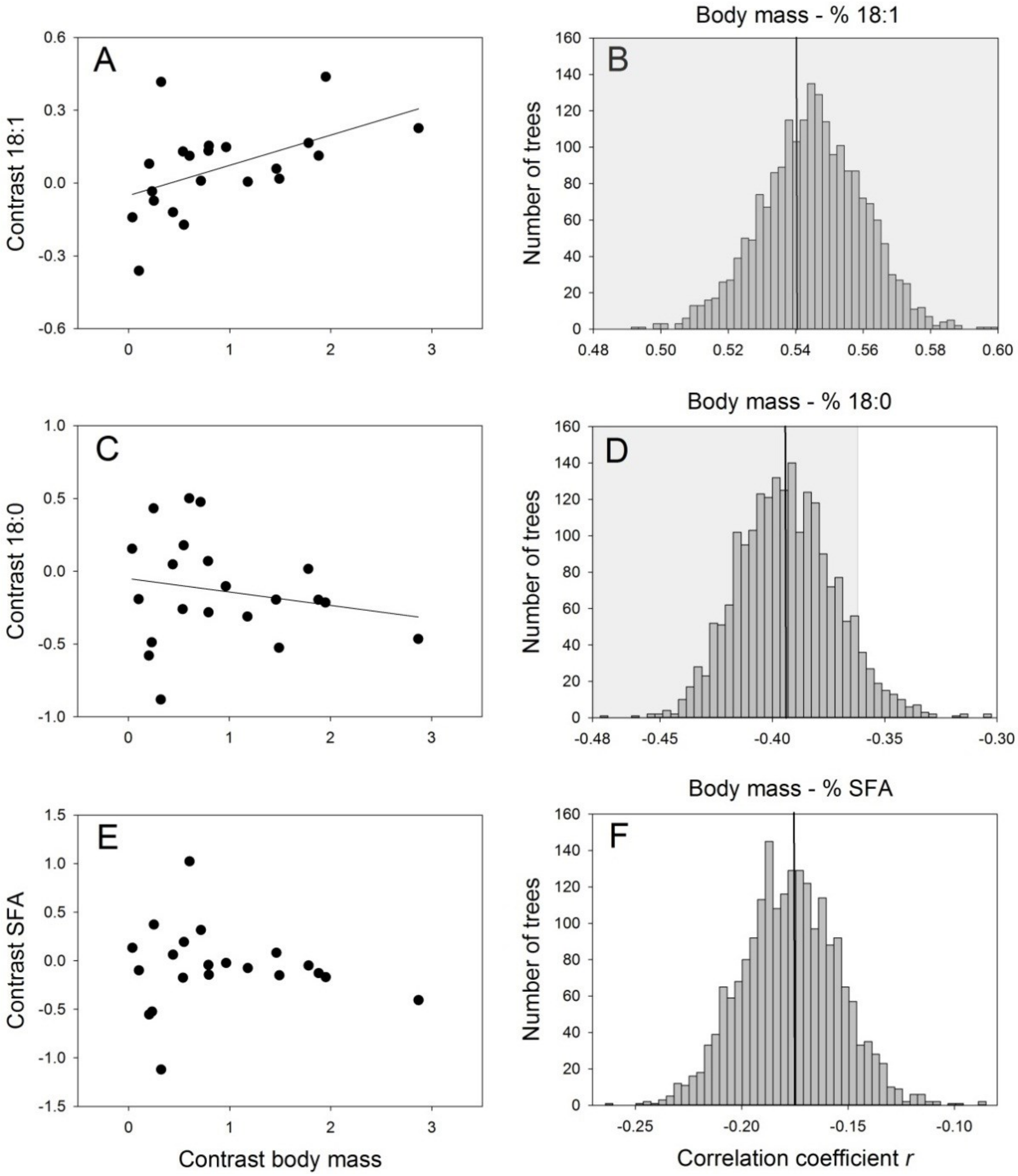

Figure 2.7. Relationship between body mass and % PL fatty acid independent contrasts obtained from *RNAPII, CO1, argK* and *EF-1alpha* phylogeny (panels A, C and E). Panels on the right (B, D and F) show the distribution of correlation coefficient resulting from analyses performed with 2041 different trees. Shaded areas represent a significant relationship for the correlation coefficient *r* (*P*<0.05, one-tailed test).

Table 2.3. Results and trends from the regression analysis of %FA abundance on body mass for NL and NEFA fractions. Fatty acids with relative abundances >5% only are presented. Significant values of *P* (<0.05) are in bold.

	NL			**NEFA**		
Fatty acid	r^2	***P* value**	**Trend**	r^2	***P* value**	**Trend**
C16:0	0.13	0.572	Slight decrease	0.11	**<0.001**	Slight decrease
C18:0	0.76	**<0.001**	Decrease	0.27	**0.03**	Decrease
C18:1 n-9	0.84	**<0.001**	Increase	0.58	**<0.001**	Increase
C18:2 n-6	0.24	**<0.001**	Decrease	0.1	**<0.001**	Slight increase
C18:3 n-3	0.49	**<0.001**	Decrease	0.12	**<0.001**	Decrease
∑PUFAs	0.48	**<0.001**	Decrease	0.001	0.655	None
∑SFAs	0.68	**<0.001**	Decrease	0.25	**<0.001**	Decrease
UI	0.04	**0.008**	Slight increase	0.08	**<0.001**	Slight increase
PI	0.45	**<0.001**	Decrease	0.004	0.415	None

Table 2.4. Probability results for the ANCOVA taking into account mass and genus for the fatty acids in the NL and NEFA fractions. Fatty acids with relative abundances >5% only are presented. Non-significant values of *P* (>0.05) are in bold.

Fatty acid	**Variables**	**NL**	**NEFA**
		***P* value**	
C16:0	Mass	0.044	0.001
	Genus	<0.001	<0.001
	Genus*Mass	<0.001	<0.001
C18:0	Mass	<0.001	<0.001
	Genus	**0.061**	<0.001
	Genus*Mass	**0.512**	<0.001
C18:1 n-9	Mass	<0.001	<0.001
	Genus	<0.001	<0.001
	Genus*Mass	<0.001	<0.001
C18:2 n-6	Mass	<0.001	**0.23**
	Genus	<0.001	<0.001
	Genus*Mass	<0.001	<0.001
C18:3 n-3	Mass	<0.001	<0.001
	Genus	<0.001	<0.001
	Genus*Mass	<0.001	<0.001
∑PUFAs	Mass	<0.001	**0.174**
	Genus	<0.001	<0.001
	Genus*Mass	<0.001	<0.001
∑SFAs	Mass	**0.356**	<0.001
	Genus	**0.154**	0.001
	Genus*Mass	0.041	<0.001
UI	Mass	<0.001	<0.001
	Genus	<0.001	<0.001
	Genus*Mass	<0.001	<0.001
PI	Mass	<0.001	<0.001
	Genus	<0.001	<0.001
	Genus*Mass	<0.001	<0.001

Table 2.5. NCBI *GenBank* accession numbers for the species used in the phylogenetic analysis.

Species	CO1	ArgK	RNAP II	EF-1 α
Aglae caerulea	EU421542		EU421289	EU421413
Apis cerana	EU163108	EU163192	EU163025	EU162861
Eufriesea anisochlora	EU421583	EU421710	EU421325	EU421456
Euglossa asarophora	EU421496	EU421628	EU421248	EU421367
Eulaema bombiformis	EU421524	EU421655	EU421271	EU421395
Euglossa bursigera	EU421573	EU421701	EU421319	EU421446
Eufriesea chrysopyga	EU421507	EU421638	EU421258	EU421378
Eulaema cingulata	EU421501	EU421633	EU421253	EU421372
Euglossa cognata	EU421510	EU421640	EU421261	EU421381
Euglossa crassipunctata	EU421494	EU421626	EU421246	EU421365
Euglossa deceptrix	EU421546	EU421675	EU421293	EU421418
Euglossa dissimula	EU421530	EU421661	EU421277	EU421401
Eufriesea dressleri	EU421552	EU421682		EU421425
Exaerete frontalis	EU421478	EU421607		EU421352
Euglossa imperialis	EU421537	EU421668	EU421284	EU421408
Eulaema meriana	EU421512	EU421642	EU421262	EU421383
Euglossa mixta			EU421309	EU421436
Eulaema nigrita	EU421555	EU421685	EU421300	EU421427
Eulaema polychroma	EU421579	EU421706	EU421324	EU421452
Eufriesea pulchra	EU421506	EU421637	EU421257	EU421377
Eufriesea rufocauda	EU421459	EU421587	EU421212	EU421328
Euglossa sapphirina	EU421499	EU421631	EU421251	EU421370
Eufriesea schmidtiana	EU421458	EU421586		EU421327
Euglossa tridentata	EU421528	EU421659	EU421275	EU421399

CHAPTER THREE – Membrane composition diversity in heterothermic insects: variation with species body mass and thoracic temperature excess

Introduction

Insects are remarkably widespread throughout the world and show incredible diversity in terms of thermal properties. Many insect species are ectothermic poïkilotherms, as their internal temperature varies, but they are unable to generate and maintain enough heat to increase their body temperature above that of the environment. Therefore, many insects are strongly dependent on the environmental temperature to sustain their daily activities. Other insect species are heterotherms, thus switching periodically between ectothermy (heat acquired from the environment) and endothermy (heat generated endogenously). These species therefore show evidence of thermoregulation, a feature that is strongly linked to the evolution of insect flight (Heinrich, 1981; Heinrich, 1995).

Thermoregulation in insects can be adjusted in response to varying needs, such as during the transition from rest to flight, or in particular behavioral situations such as basking, brood incubation or nest defense. Nonetheless, the ability or inability to regulate body temperature remains a diverse and important, albeit misunderstood subject in insect physiology. Flies, bees, wasps and beetles are considered to be more evolutionarily advanced flyers among insects, and are able to contract their flight muscles in response to both action potentials and stretching. Large flying insects, such as bees, are a remarkable example of a group with different capacities to thermoregulate, more strongly linked to body mass than to phylogenetic origin (Heinrich, 1995). The thermal properties of their environment constrain their activities, because many bee species require an elevated body temperature to achieve flight, their essential means of locomotion.

Warming up for flight may involve different mechanisms, from basking using a specific body posture to shivering thermogenesis, or a mix of both. Some bee species use the latter strategy and are able to elevate their thoracic temperature by rapidly contracting their flight muscles. During flight, muscles operate in an asynchronous mode, as the contraction of the down-stroke muscles automatically stretches the up-stroke muscles that trigger contraction by stretch-activation, and vice-versa. This mechanism is known as the myogenic flight system (Heinrich, 1995). On the other hand, during shivering, muscles do not operate myogenically, but are rather contracting in response to neural stimulation and the opposite sets of muscles contract simultaneously in a tetanus (Esch and Goller, 1991). Muscles are partially or totally declutched from the wings, resulting in no apparent movement of the wing and only slight deformations of the thorax, with slow and long contractions during warm-up (Heinrich, 1995). These tetanic contractions of the dorsoventral and dorsal longitudinal indirect flight muscles generate sufficient heat during pre-flight warm-up, elevating the bee's thoracic temperature above the environmental temperature (Willmer and Stone, 2004). Bees face the problem of overheating, as thermogenesis associated with flight can lead to excess heat, but enhanced convective heat loss by shunting haemolymph to the abdomen ensures thermal balance. Other bees can adjust their temperature behaviorally by searching for shade or restraining their activities to specific periods of the day, or by dissipating their heat excess through the evaporation of collected water (Heinrich, 1995). Large flying insects thus display features of regional and temporal heterothermy, the thorax muscles being the main tissue capable of generating heat.

Traits related to thermoregulation at the interspecific level, such as rates of radiative heat gain, endothermic heat generation and heat loss can be predicted by size and phylogeny. Moreover, endothermy is not restricted to some temperate bee genera, but occurs in most species with a body mass above 35-50 mg (Willmer and Stone, 2004). Social species not only use their thermoregulatory ability for flight, but have evolved more ways of profiting of their elevated temperatures. As such, honeybees and bumblebees have been shown to maintain high nest or swarm temperatures (~30°C), even when outside temperature falls to negative values (Stabentheiner, 2003). Queen bees use their heating capacities to incubate the brood, and both bumblebees and honeybees warm up when presented with high quality food (Willmer and Stone, 2004; Mapalad *et al.*, 2008). The most sophisticated example of the use of this heating apparatus can be seen in Japanese honeybees defending their nests against attacks from giant hornets (Ono *et al.*, 1995). Endothermy is energetically costly, but reaching the appropriate muscle temperature to fly is essential for these winged insects.

Interestingly, there is important interspecific and intraspecific variation in endothermy and thermoregulatory capacity in bees (Stone and Willmer, 1989; Stone, 1994; Willmer and Stone, 2004), but whether this variation has consequences on the cellular properties of the thoracic flight muscle has not been investigated to date. Within a species, high-altitude populations can be more endothermic than populations at sea level and are consequently able to forage at lower ambient temperatures, as seen in the tropical bee *Amegilla sapiens* (Stone, 1993). Temperate bee species with poor thermoregulatory capacities rely on behavioral strategies, such as long periods of basking as seen in the genus *Andrena* (Herrera, 1995). Dyer and Seeley (1987) noticed interspecific differences in endothermic heat generation in four species of honeybees (genus *Apis*) that they linked with differences in nesting behaviour and colony demography. Bee species clearly differ in their ability to thermoregulate, but whether or not this variation has implications at the cellular membrane level remains unknown.

Membranes are sensitive to temperature, and organisms ranging from bacteria to poïkilothermic animals can modify their membranes to adjust to their thermal environment in a process named homeoviscous adjustment (HVA) (Sinensky, 1974; Hazel and Williams, 1990). Various studies have looked at how organisms respond to changes in environmental temperature by altering their membrane composition. Mechanisms often used include phospholipid head group restructuring (Phosphatidylcholine/Phosphatidylethanolamine ratio), cholesterol content (to a lesser extent), and most importantly changes in acyl chain composition (Hazel and Williams, 1990). The latter mechanism has received much attention lately. The rationale behind changes in acyl chain composition lies in the alteration of the physical environment of the membrane. Introducing a *cis* double bond in an acyl chain can significantly affect the molecular and physical characteristics of a phospholipid. This double bond induces a 30° bend in the carbon chain, giving a broader conformation to the unsaturated fatty acid (UFA) as opposed to the saturated fatty acid (SFA). This expansion prevents a closer packing of the PL and a decrease in their hydrophobic bulkiness in the event of a decrease in temperature of the membrane (Hazel and Williams, 1990). Thus, an elevated unsaturation can keep the expanded nature of the hydrophobic region of the membrane. However, this is not a direct and simple relationship, as the number and placing of the double bond can restrict the conformation available to the acyl chain. Moreover, different types of fatty acids possess different melting points that vary according to the length of the carbon chain or the number and position of the double bond (Hazel and Williams, 1990). Therefore, predicting the exact effect of various types of acyl chains in the membrane is not an easy task. There is, however, a remarkable trend of accumulating long-chain polyunsaturated fatty acids (PUFAs) in cold-exposed or winter-active poïkilotherms (Hazel and Williams, 1990).

The fatty acid composition of the membrane has been shown to be linked with habitat-specific temperatures, as species of vertebrates living in cold climates generally possess more unsaturated membranes than species found in warmer environments (Logue *et al.*, 2000). Moreover, this remodeling of cellular membranes with temperature has been established in a wide variety of organisms, from bacteria and plants to metazoans (Uemura *et al.*, 1995; Ayala-Del-Río *et al.*, 2010). In teleost fish, a heat stress can affect the regulation of genes linked to fatty acid metabolism (Buckley, 2006), and membrane saturation can vary with season (Guderley, 2004; Kraffe *et al.*, 2007). In reptiles, membranes are also subject to changes in polyunsaturation such as in cold-acclimated crocodiles (Seebacher *et al.*, 2009). Some invertebrate studies have also looked at changes in membrane composition in the context of homeoviscous adjustments. Fruit flies (*Drosophila melanogaster*) subjected to rapid cold hardening, a gradual cooling known for its protective effect in several species of insects, were found to respond by reductions in membrane SFA and MUFA, and increases in PUFA (Overgaard *et al.*, 2005). An appropriate phospholipid fatty acid composition of membranes appears to be crucial to the survival to freezing temperatures in many organisms such as the freeze-tolerant earthworm *Dendrobaena octaedra*. When exposed to low copper concentrations, it experiences a significant decrease in the amount of membrane PUFA 18:2 n-6,9 accompanied by an increase in lipid peroxidation, reducing the freeze tolerance and affecting the mortality at higher freezing temperatures (Bindesbol *et al.*, 2009). Globally, it appears that the cellular thermal environment greatly influences membrane composition in a variety of organisms.

Question and predictions

This study investigates how the diversity in thermoregulatory capacity found in bees and wasps affects the membrane phospholipid composition of flight muscle tissue. We will test the relationships between thoracic temperature excess (the gradient between thoracic surface and ambient air temperature) and the thoracic fatty acid composition in North American bee and wasp species. We expect to find higher proportions of PUFAs at lower thoracic temperature excess, while muscles operating at higher temperature excess should contain more MUFAs and SFAs.

Materials and methods

Field sampling of local Hymenopterans

Individuals from various species of Hymenopterans were sampled during the spring and summer of 2011 and 2012 using insect nets at various locations in the Ottawa area. A total of 130 individuals were sampled, pertaining to 15 genera and three different superfamilies of the Hymenoptera order (see Table 2.1 for a complete list). Body masses of the collected specimens ranged from 31 to 550mg (see Table 2.2). Individuals were quickly transferred to a restraining device (50mL syringe with netting at the open end) to immobilize them while measuring their thoracic surface temperature (T_{th}). Thermograms were taken in the shade using an infrared camera (EX300, FLIR systems). Individuals were then transported in 50mL transparent tubes, placed on ice in a cooler until arrival at the lab where they were stored in the freezer at -20°C. Within 24 to 48h, specimens were identified to the species level (when not possible, to the subgenus level) using various keys (Michener, 2007;

Buck *et al.*, 2008); their body parts separated, weighted and stored in 1.5 mL CryoVials at -80°C. Thermographic data was stored on a computer before further analysis and extraction of the T_{th}. An Enviro-meter (Fisher scientific) was used to measure the ambient air temperature (T_a). Relative humidity, solar radiation and wind speed were also measured at each sampling site. The thorax temperature excess, the gradient between thoracic surface and ambient air temperature (T_{th}-T_a) was used as a measure to assess the endothermic capacity of the collected specimens (Kovac and Stabentheiner, 2012).

Lipid analysis

Whole-thorax lipids were first extracted using a 2:1 mixture of chloroform and methanol (Folch *et al.*, 1957) and homogenized with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland). A fraction of the homogenate (5mL) was taken for immediate analysis; while the rest was stored in glass tubes at -20C. Samples were then centrifuged (10mins at 3000 RPM) and filtered in three successive steps; water was removed by adding 2.5 mL of KCl (33 mM) and the aqueous phase was eliminated with a water-powered vacuum pump. After evaporation on a Büchi RE 121 rotary evaporator from Brinkman Instruments Inc. (Westbury, NY, USA), samples were re-suspended in 6mL of a 3:2 mix of hexane and isopropanol; then stored in the freezer at -20°C until lipid separation was performed.

Separation of lipids was done following a protocol described in Magnoni and Weber (2007). Lipid fractions of neutral lipids (NL), non-esterified fatty acids (NEFA) and phospholipids (PL) were separated by filtration on 1ml/100mg Supelclean solid-phase extraction tubes (Sigma, St-Louis, Missouri, USA). Heptadecanoic acid (17:0; 30mg/100ml hexane) was added to each fraction to be used as an internal standard since preliminary experiments had shown that this fatty acid (FA) is absent from NEFA, NL and PL fractions of bee tissues. Following the separation, the NEFA fraction was methylated using a mix of methanol, dimethoxypropane and concentrated HCl (1M). The NL and PL fractions were transesterified using 2mL of a solution of acetyl chloride (1M) in methanol and heated at 90°C for 2 hours. The three fractions were then evaporated, re-dissolved in 50µL of isooctane and transferred to gas chromatograph autosampler vials.

The fractions were analyzed on an Agilent technologies HP 6890 series gas chromatograph system equipped with a flame-ionization detector and a 60 m DB-23 fused silica capillary column (internal diameter: 0.25mm and 0.25µm film; J&W Scientific, Folsom, CA, USA). The temperature program of the column consisted of 1 min at 130°C after injection, then an increase of 6.5°C.min-1 to a temperature of 170°C. Immediately after, an increase of 2.75°C.min-1 was applied to reach 215°C maintained during 12mins. The temperature was then raised at a rate of 40°C.min-1 to reach 230°C, a temperature which was subsequently maintained during 3 min. The total run time for each sample was of 38.89 min. Individual fatty acids were identified by determining exact retention time with authentic standards (Supelco, Bellefonte, PA, USA). The relative abundance of individual fatty acids was measured using the surface area under the peak and correcting for the internal standard (17:0) in the ChemStation software environment (Agilent Technologies, Wilmington, DE, USA).

Data analysis

All statistical analyses were performed using the Systat 13 software. All values are presented as mean ± SE. Dependent and independent variables were first tested for normality using the Shapiro-Wilk test. We also calculated two indices related to the level of unsaturation of the membrane, namely unsaturation index (UI) and peroxidation index (PI) using the definitions found in (Hulbert *et al.*, 2007). UI represents the number of double bonds per 100 acyl chains and is calculated as UI = (% monoenoics) + (2*% dienoics) + (3* % trienoics) + (4*% tetraenoics)+ (5*% pentaenoics) + (6*% hexaenoics). PI indicates the susceptibility of fatty acids to peroxidative damage and is calculated with the following formula: PI = (0.025*% monoenoics) + (1*% dienoics) + (2*% trienoics) + (4*% tetraenoics) + (6*% pentaenoics) + (8*% hexaenoics). Relationships between body mass and FA relative abundance in birds and mammals follow power functions (Hulbert *et al.*, 2007). Therefore, variables were log-transformed to linearize the data. Normality of residuals was also verified using the Shapiro-Wilk test, and homoscedasticity was assessed using Levene's test.

For each fatty acid, the relationships with body mass or thoracic temperature excess (T_{th}-T_a) were first analyzed separately using linear regressions, and followed by analyses of covariance (ANCOVA) where body mass, thoracic temperature excess and superfamily were included in the model. For these analyses, only two superfamilies (Apoidea-Anthophila and Vespoidea) were analyzed, given sample size available. Values were weighted as a function of the sample size (N). We first analyzed the model with all the interaction terms, and removed non-significant interactions for the final model. Finally, we presented correlations between thoracic temperature excess and membrane composition independent of body mass, by performing analyses on the residuals obtained from significant regressions with body mass.

Results

Thoracic temperature excess variation with body mass

Among the Hymenopteran species collected, we found a significant and positive increase in thoracic temperature excess (T_{th}-T_a) with body mass (r^2=0.54, P<0.001; Fig. 3.2). An ANCOVA including superfamily as a covariate showed a significant influence of body mass and superfamily (Mass: $F_{(1,106)}$=112.446, P<0.001; Superfamily: $F_{(1,106)}$=40.733, P<0.001). Further analysis on superfamilies showed that these increases with body mass remained significant among Apoidea (Anthophila) (r^2=0.64, P<0.001), but not among Vespoidea (r^2=0.08, P=0.128). Species of bees (Apoidea-Anthophila) had a higher thoracic temperature excess than wasps species (average T_{th}-T_a for Apoidea: 14.02°C; Vespoidea: 10.07°C).

Fatty acids in the phospholipid fraction of the thorax in local Hymenopterans

The thoracic membrane phospholipid fraction of the 27 Hymenopterans species was composed of five major fatty acids, where 16:0 and 18:0 were the main saturated fatty acids (SFA), 18:1 (n-9) was the main monounsaturated fatty acid (MUFA), 18:2 (n-6) and 18:3 (n-3) were the main polyunsaturated fatty acids (PUFA). Other fatty acids were found in relatively low abundances (<1%) and their presence varied among individuals of a species: 14:0, 16:1 (n-7), 20:0, 20:1, 21:0, 22:0, 20:5, 22:1, 22:6 and 24:0.

The relative abundance of various fatty acids in the phospholipid fraction changed significantly with T_{th}-T_a and with body mass. Oleic acid abundance, 18:1 (n-9), increased significantly with body mass (r^2=0.27, P<0.001; Fig. 3.3A), and with T_{th}-T_a (r^2=0.23, P<0.001; Fig. 3.3B) when analyzed separately. When both independent variables were analyzed with the Superfamily as a factor, the results from the ANCOVA showed an interaction between Mass, T_{th}-T_a and Superfamily (Mass: $F_{(1,103)}$=2.843, P=0.095; T_{th}-T_a: $F_{(1,103)}$=10.660, P=0.001; Superfamily: $F_{(1,103)}$=1.119, P=0.293; Superfamily*Mass: $F_{(1,103)}$=7.302, P=0.008; Superfamily*Mass*T_{th}-T_a: $F_{(1,103)}$=7.042, P=0.009). Thus, the relationship between 18:1 content, body mass and thoracic temperature excess differs between superfamilies. The differences between superfamilies were further analyzed by performing multiple regressions on each superfamily separately. 18:1 increased significantly with T_{th}-T_a in Apoidea (Anthophila) but not with mass (r^2=0.51; Mass: P=0.107; T_{th}-T_a: P<0.001). Among Vespoidea, there was no significant relationship between 18:1 and T_{th}-T_a or body mass (r^2=0.13; Mass: P=0.615; T_{th}-T_a: P=0.079). Overall, we found a significant effect of thoracic temperature excess in Apoidea and a similar tendency in Vespoidea.

Linoleic acid, 18:2 (n-6), did not vary with body mass (r^2=0.005, P=0.453; Fig. 3.4A), nor with thoracic temperature excess (r^2=0.01, P=0.184; Fig. 3.4B). Results from the ANCOVA showed a significant interaction between superfamily and body mass (Mass: $F_{(1,104)}$=0.317, P=0.575; T_{th}-T_a: $F_{(1,104)}$=0.955, P=0.331; Superfamily: $F_{(1,104)}$=0.241, P=0.625; Superfamily*Mass: $F_{(1,104)}$=6.909, P=0.01). This could explain the fact that Apoidea (Anthophila) seem to have less abundance of 18:2 when mass increases, while in Vespoidea there seems to be the opposite trend. Indeed, multiple regression analysis showed a nearly significant relationship with T_{th}-T_a (r^2=0.25; Mass: P=0.233; T_{th}-T_a: P=0.054) in

Apoidea (Anthophila). In Vespoidea, there was no significant relationship with T_{th}-T_a, or body mass (r^2=0.07; Mass: P=0.280; T_{th}-T_a: P=0.584).

The relative abundance of alpha-linolenic acid, 18:3 (n-3), did not vary significantly with body mass (r^2=0.03, P=0.055; Fig. 3.4C) among the Hymenopteran species studied. It did, however, decrease significantly with the thoracic temperature excess but only explained a small amount of variation (r^2=0.04; P=0.001; Fig. 3.4D). Species of predatory wasps (Apoidea-Spheciformes) had trace amounts of 18:3 (<1%) and were therefore not included in the analysis. Results from the ANCOVA revealed a significant effect of temperature excess and superfamily (Mass: $F_{(1,102)}$=1.257, P=0.256; T_{th}-T_a: $F_{(1,102)}$=4.137, P=0.044; Superfamily: $F_{(1,102)}$=4.107, P=0.045). Multiple regression in Apoidea (Anthophila) showed no significant changes in 18:3 with body mass or T_{th}-T_a (r^2=0.03; Mass: P=0.178; T_{th}-T_a: P=0.134). However, there was a significant increase in 18:3 abundance with body mass in Vespoidea (r^2=0.17; Mass: P=0.046; T_{th}-T_a: P=0.086). We therefore cannot identify strong and consistent effects of mass, temperature or superfamily for 18:3.

The abundance of total PUFAs did not vary significantly with body mass (r^2=0.03, P=0.07; Fig. 3.4E), but it decreased significantly with increasing T_{th}-T_a, explaining a small proportion of variation (r^2=0.03, P=0.049; Fig. 3.4F). Results from the ANCOVA revealed an interaction between superfamily, body mass and temperature excess (Mass: $F_{(1,102)}$=1.558, P=0.215; T_{th}-T_a: $F_{(1,102)}$=5.348, P=0.023; Superfamily: $F_{(1,102)}$=0.251, P=0.617; Superfamily*Mass: $F_{(1,102)}$=9.162, P=0.003; Superfamily*Mass*T_{th}-T_a: $F_{(1,102)}$=5.507, P=0.021). Therefore, the relationship between PUFA abundance and temperature excess combined with body mass differs between superfamilies. This is reflected in the multiple regression analysis which showed a significant decrease of PUFA with T_{th}-T_a but not with body mass in Apoidea (Anthophila) (r^2=0.20; Mass: P=0.747; T_{th}-T_a: P=0.006). Moreover, in Vespoidea PUFA increased with body mass but not with T_{th}-T_a (r^2=0.28; Mass: P=0.003; T_{th}: P=0.161). The increase in PUFA with mass in Vespoidea can be explained by the fact that they possess up to three times more 18:3 than 18:2, and that the abundance in the former increases with mass. However, Apoidea have similar abundances of the two polyunsaturates, and they tend to decrease with mass or temperature excess. Therefore, there are important differences in polyunsaturates between the two superfamilies.

The abundance of palmitic acid, 16:0, decreased significantly with body mass (r^2=0.60, P<0.001; Fig. 3.5A) and with T_{th}-T_a (r^2=0.69, P<0.001; Fig. 3.5B) in our Hymenopteran species. Results from the ANCOVA showed significant interactions between body mass, T_{th}-T_a and superfamily (Mass: $F_{(1,102)}$=40.838, P<0.001; T_{th}-T_a: $F_{(1,102)}$=55.606, P<0.001; Superfamily: $F_{(1,102)}$=7.318, P=0.008; Superfamily*Mass: $F_{(1,102)}$=6.915, P=0.01; Superfamily*T_{th}-T_a: $F_{(1,102)}$=11.748, P<0.001; Superfamily*Mass*T_{th}-T_a: $F_{(1,102)}$=13.510, P<0.001). Multiple regressions performed on each group revealed that 16:0 decreased significantly with T_{th}-T_a and body mass in Apoidea (Anthophila) (r^2=0.72; Mass: P=0.001; T_{th}-T_a: P<0.001). Similarly, 16:0 decreased significantly with T_{th}-T_a and body mass in Vespoidea (r^2=0.76; Mass: P<0.001; T_{th}-T_a: P<0.001).

The relative abundance of stearic acid, 18:0, decreased significantly with body mass (r^2=0.14, P<0.001; Fig. 3.5C) and with T_{th}-T_a (r^2=0.13, P<0.001; Fig. 3.5D). The ANCOVA indicated a significant interaction between superfamily and body mass (Mass: $F_{(1,104)}$=8.460, P=0.004; T_{th}-T_a: $F_{(1,104)}$=9.225, P=0.003; Superfamily: $F_{(1,104)}$=15.174, P<0.001; Superfamily*Mass: $F_{(1,104)}$=10.644, P=0.001). This could be explained by lack of clear trends among Apoidea (Anthophila) with either temperature excess or body mass, as opposed to Vespoidea. Indeed, multiple regressions showed no significant trends between 18:0 and T_{th}-T_a or body mass in Apoidea (Anthophila) (r^2=0.11; Mass: P=0.059; T_{th}-T_a: P=0.871). In

Vespoidea, however, 18:0 decreased with T_{th}-T_a and body mass (r^2=0.94; Mass: P<0.001; T_{th}-T_a: P<0.001).

The sum of saturated fatty acids, SFA, also decreased significantly with body mass (r^2=0.62, P<0.001; Fig. 3.5E) and with T_{th}-T_a (r^2=0.60, P<0.001; Fig. 3.5F). Results from the subsequent ANCOVA showed interactions between body mass, temperature and superfamily (Mass: $F_{(1,103)}$=52.950, P<0.001; T_{th}-T_a: $F_{(1,103)}$=84.649, P=0.001; Superfamily: $F_{(1,103)}$=6.565, P=0.004; Superfamily*T_{th}-T_a: $F_{(1,103)}$=28.228, P<0.001; Superfamily*Mass*T_{th}-T_a: $F_{(1,103)}$=7.964, P=0.006). Multiple regression analysis confirmed this decrease in SFA with T_{th}-T_a and body mass in Apoidea (Anthophila) (r^2=0.73; Mass: P<0.001; T_{th}-T_a: P<0.001). In Vespoidea, SFA also decreased with T_{th}-T_a and with body mass (r^2=0.89; Mass: P<0.001; T_{th}-T_a: P<0.001).

Unsaturation index, the number of double bonds per 100 acyl chains, did not vary significantly with body mass (r^2=0.003, P=0.566; Fig. 3.6A) or T_{th}-T_a (r^2=0, P=0.925; Fig. 3.6B). An ANCOVA with superfamily as a covariate showed a difference between superfamilies (Mass: $F_{(1,105)}$=1.608, P=0.208; T_{th}-T_a: $F_{(1,105)}$=2.356, P=0.128; Superfamily: $F_{(1,105)}$=28.839, P<0.001). The results from a multiple regression showed no significant changes in UI among Apoidea (Anthophila) (r^2=0.04; Mass: P=0.43; T_{th}-T_a: P=0.139). However, UI increased significantly with body mass, but not T_{th}-T_a, in Vespoidea (r^2=0.41; Mass: P<0.001; T_{th}-T_a: P=0.486).

The sensitivity of phospholipids to peroxidative damage, peroxidation index, did not vary with body mass among our Hymenopteran species (r^2=0.008, P=0.310; Fig. 3.6C), but it decreased significantly with T_{th}-T_a (r^2=0.04, P=0.03; Fig. 3.6D). The subsequent ANCOVA revealed an effect of temperature excess and superfamily (Mass: $F_{(1,105)}$=1.637, P=0.204; T_{th}-T_a: $F_{(1,105)}$=11.076, P=0.001; Superfamily: $F_{(1,105)}$=24.365, P<0.001). Multiple regression analysis showed that PI decreased significantly with T_{th}-T_a, but not mass, in Apoidea (Anthophila) (r^2=0.13; Mass: P=0.401; T_{th}-T_a: P=0.01). On the other hand, PI increased significantly with mass, but not T_{th}-T_a in Vespoidea (r^2=0.26; Mass: P=0.004; T_{th}-T_a: P=0.099).

Residual analysis to account for the effects of mass on membrane composition and temperature excess

Finally, to represent the relationships between fatty acid composition and thoracic temperature excess, we further analyzed data using residuals obtained from the regressions with body mass.

The increase in 18:1 with T_{th}-T_a remained significant and positive when correcting for the effect of body mass on both variables (r=0.39, P<0.001; Fig. 3.7A). In polyunsaturated fatty acids, the decrease in 18:3 with T_{th}-T_a also stayed significant when residuals of temperature excess were plotted (r=0.19, P=0.044; Fig. 3.7B), as did the decrease in total PUFAs (r=0.28, P=0.004; Fig 2.7C). As for saturated fatty acids, 16:0, 18:0 and combined SFA abundance decreased with T_{th}-T_a (r=0.52, 0.22 and 0.55, respectively; P<0.001, 0.02 and 0.001, respectively; Fig. 3.8A-C). Finally, residual peroxidation index decreased significantly with residual T_{th}-T_a (r=0.29; P=0.002; not shown).

Discussion

The scaling of membrane composition with temperature excess and body mass in Hymenopterans

A major finding of this study is that membrane composition in various species of Hymenopterans changes systematically with thoracic temperature excess, a proxy of the thermoregulatory ability of the flying insect. We found that membrane phospholipid composition across 3 superfamilies and 15 genera of the Hymenoptera order consisted of essentially five major fatty acids. The relative abundances of these fatty acids varied with thoracic temperature excess in the directions predicted by the Homeoviscous Adaptation theory (HVA) (Sinensky, 1974; Hazel, 1995), with a few particularities. We found that thoraxes working at a lower temperature excess contained significantly more 18:3 (n-3) and total PUFAs than those of highly endothermic species (Fig. 3.4D and F), even when controlling for the effects of body mass on temperature excess (Fig. 3.7B and C). Compensating for this decrease in PUFAs with temperature is the significant increase in the MUFA 18:1 (n-9) (Fig. 3.3B) with thorax temperature excess; somewhat independent of body mass (Fig. 3.7A). Contrary to our initial predictions, the amounts of SFAs (16:0 and 18:0) decreased with thoracic temperature excess (Fig. 3.5B, D and F), again independently of body mass (Fig. 3.8A, B and C). This could be due to the length of the SFA 16:0, as the HVA theory also predicts shorter fatty acid chain lengths at lower temperatures.

Our study suggests that for heterothermic insects with varying levels of endothermy to ectothermy, acyl composition of the membrane is adapted to provide or maintain muscle functionality. Flying Hymenopterans offer an interesting model because of their varying capacity to thermoregulate, and they are therefore in a “grey zone” in terms of thermal strategies. It is interesting to compare our results with what is known in other poikilothermic animals. In ectothermic vertebrates from cold environments or facing a change in temperature due to seasonality, the general trend of membrane acyl composition adjustment seems to involve increases in PUFA with cold temperatures while MUFA remain unchanged (Logue *et al.*, 2000; Guderley, 2004; Seebacher *et al.*, 2009). However, these modifications of the acyl chain of the membrane seem to differ as a function of many factors such as the directionality of the temperature change, tissue, and class or subclass of phospholipids (Kraffe *et al.*, 2007). In ectothermic invertebrates (such as most insects), increases in PUFA abundance accompanied by decreases in MUFA and SFA seem to be the norm when subjected to cold environment (Cuculescu *et al.*, 1995; Overgaard *et al.*, 2005; Bindesbol *et al.*, 2009). However, results deviating from the predictions of the HVA theory were observed in some insects, as low temperature acclimation has been found to trigger increases in MUFA and SFA without changes in PUFA (Goto and Katagiri, 2011), or increases in MUFA and PUFA depending on the class of PL (Izumi *et al.*, 2009). Globally, increased unsaturation of the acyl chain with cold environment appears to be a common mechanism for poïkilotherms, although the directionality of the changes and nature of the fatty acids involved seem to be context specific.

The increased unsaturation of the membrane lipids has been shown to promote survival at cold temperature in various species (Hazel, 1995). However, unsaturation index (the number of double bonds per 100 acyl chains), remained unchanged with thoracic temperature excess in our Hymenoptera species (Fig. 3.6B). This is because 18:1, the most abundant fatty acid in the membrane (ranging from 37% in *Andrena* sp. to 73% in *Apis mellifera*; Fig. 3.3B) increased significantly with temperature while the second most abundant group of fatty acids, PUFAs, decreased significantly (from 43% in *Andrena* sp. to 11% in *Apis mellifera*; Fig. 3.4F). The two PUFAs (18:2 and 18:3) have a more important weight in the calculation of UI and end up cancelling the effect of the increase in 18:1, resulting in no variation overall. Another aspect that seems to deviate from the HVA theory is the decrease in SFA with temperature. However, this trend could be understood from the point of view of chain length rather than saturation, because exposure to cold temperature often elicits reduction in the average length of the acyl chain (Hazel and Williams, 1990). 16:0 is the shortest fatty acid found amongst the important fatty acids in the thorax membrane, nearly doubling the abundance of the other SFA, 18:0 (Fig. 3.5B and D). The abundance of 16:0 spans from 5% (*Bombus bimaculatus*) to 19% (*Nomada ruficornis*), while 18:0 varies only from 3 to 9% (*Andrena Scrapteropsis* sp. to *Osmia lignaria*). Moreover, our results show that the decrease in SFA is the strongest among all fatty acids studied, and temperature excess explains most of the variation in SFA (Fig. 3.5F). It is therefore plausible that shorter chain length and higher polyunsaturated membranes are part of the strategy to maintain homeostasis of the membrane in weakly endothermic to ectothermic Hymenopterans.

Another finding of this study is the systematic change in membrane composition with body mass in Hymenopterans. The significant increase of 18:1 (Fig. 3.3A), the lack of significant variation in 18:2 and total PUFA (Fig.3.4A, E), and the significant decrease in 18:0 abundance (Fig. 3.5C) are similar to what was found among various species of Euglossine bees (see Chapter 2). The positive relationship between 18:1 and body mass was in agreement with the "membrane pacemaker" theory of metabolism, that describes body mass-fatty acid composition variations in mammals and birds (Hulbert, 2003). Contrary to Hulbert and Else's study on endothermic vertebrates, our dataset included Hymenopterans with varying thermoregulatory abilities, and these variations were in fact found to be influenced by thoracic temperature excess rather than body mass in 18:1 and 18:0 for bees

(Apoidea-Anthophila). The lack of a significant negative relationship between PUFAs and body mass does not agree with the findings of Hulbert and Else on endothermic vertebrates, although there seemed to be an almost significant trend in our dataset (Fig. 3.4E). The reason behind this absence of a clear trend between PUFAs and body mass is the fact that, contrary to what was found in Euglossine bees, 18:3 (n-3) does not vary significantly with body mass (Fig. 3.4C). In fact, this polyunsaturated fatty acid was not found in all species collected, and was virtually absent (less than 1%) in Spheciformes, a group of predatory wasps closely related to bees (Anthophila) (Michener, 2007). It was also found in small quantities (under 5%) in the small bee *Nomada ruficornis*, and its abundance increased with mass in Vespoidea. Moreover, we found a high variability in 18:2 abundance with body mass (as seen in Euglossine bees), which raises questions as to a possible source of variation such as the influence of dietary availability. As for SFAs, they offered another new diverging relationship with regards to the "membrane pacemaker" theory, as 16:0, 18:0 (in Vespoidea) and total SFAs decreased significantly with body mass (Fig. 3.5A, C and E).

Altogether, these results suggest that within the array of species sampled, variation in membrane composition is influenced more strongly by thoracic temperature excess than by body mass. We have found changes in the fatty acid composition of the thoracic membrane that follow predictions from the HVA and "membrane pacemaker" theories, with a few particularities. Moreover, body mass and temperature are related in flying insects, and modification of membrane composition has to be analyzed in the scope of its functional implications for these invertebrates.

Scaling of thermoregulation with body mass in bees and wasps, and sources of variation of membrane composition

Previous studies have shown a close link between temperature and body mass in Hymenopterans (Heinrich, 1995; Willmer and Stone, 2004; Kovac and Stabentheiner, 2012). This relationship has been demonstrated at the superfamily (Apoidea), genus (*Anthophora*) and even species (*Anthophora plumipes*) levels by Stone (1994). Our results on randomly sampled Hymenopteran species confirm this relationship (Fig. 3.2). The general rule is that very small bees are poor thermoregulators (hence primarily ectothermic), whereas large bees are very good thermoregulators (hence highly endothermic). This difference appeared clearly in our dataset, where Apoidea species under 50mg have a relatively low T_{th}-T_a (generally under 10°C), whereas bigger species of bees have an elevated temperature excess (up to 20.8°C in *Bombus bimaculatus*; Fig. 3.2, blue circles). There are ecological and life-history consequences to these discrepancies: larger bees are known to be more active during the early and late hours of the day or earlier in the season, whereas small bees are more common in the middle of the day or in the hotter months of the year (Willmer and Stone, 2004). We observed these differences during our sampling throughout the spring and summer (bumblebees, *Bombus* sp. were caught early in the season and in the morning, while smaller bee species were abundant later in the day and summer). However, bees show wide variety in morphology and distribution, and this body mass-thoracic temperature relationship is not as straightforward as it seems. In fact, for a given body mass, some bee species can be more endothermic than others. It is the case of bees active in cooler climates which have higher warm-up rates and temperatures in flight, or of bees pertaining to the same genus or species (Stone and Willmer, 1989; Stone, 1994). Species of the *Bombus* and *Apis* genera offer an interesting example of deviation from this mass-temperature relationship: workers of *Bombus rufocinctus*, *Bombus bimaculatus*, *Bombus griseocollis* (Bumblebees), or *Apis mellifera* (Honeybees) had an elevated T_{th}-T_a despite their relatively small mass (around

100mg, up to 4 times smaller than their queens; see Table 2.2 and Fig. 3.2). These are highly eusocial species and their thermoregulatory ability is also useful for many behavioral tasks inside the hive. These similarities in thermoregulation between castes was previously reported by Heinrich and Heinrich (1983). The variation in endothermic ability despite similar body mass is also seen in solitary bees of the genus *Anthophora*, where endothermic abilities seem to have diverged as a response to differences in the thermal environment of the bee and as a consequence of coevolution with plants with different nectar secretion patterns (Stone, 1994). In observing the possible implication of relatedness among species, Bishop (1999) showed that thermoregulatory ability was not strongly influenced by phylogenetic history in Alaskan bees. Stone and Willmer (1989) suggested that warm-up rates are a property of the flight machinery of each species and are not simply determined by mass or surface/volume ratios. In fact, we have shown that another factor could come into play: the nature of the thoracic membrane phospholipid composition, which changes with temperature excess, independently of body mass.

Our results showed that the general body mass-temperature relationship for flying insects was not observed in wasps of the superfamily Vespoidea (Fig. 3.2, red circles). This agrees with the observations made by Kovac and Stabentheiner (2012), who obtained no simple linear correlation between T_{th} and body mass. In water foraging wasps, there are striking differences between species of the genus *Vespula* and *Polistes*: despite their relatively similar body mass, the former show high endothermic capacity, whereas the latter are weakly endothermic (Kovac *et al.*, 2009). This could be seen in our dataset (Fig. 3.2, red circles), where *Vespula* species had an elevated T_{th}-T_a (13.5 to 16.1°C), whereas *Polistes fuscatus* and *Polistes dominula* had a much lower T_{th}-T_a (6.3 to 7.4°C). Interestingly, there are also differences between genera for *Polistes*. Indeed, *P. dominula* maintains a higher temperature during flight, whereas *P. fuscatus* is considered a better thermoregulator (somewhat seen in our results as the latter has a higher temperature excess). This feature is thought to explain their different invasive capacities (Weiner *et al.*, 2011). Generally, it seems the critical mass for pronounced endothermic performance is of 50mg in wasps, and that above 200mg, there are no appreciable differences in thermoregulatory capacity. In fact, despite a fivefold difference in body mass, hornets (*Vespa crabro*) and wasps (*Vespula* sp.) were found to be capable of high and similar thermoregulation (Kovac and Stabentheiner, 2012). Moreover, the values obtained for thoracic temperature excess of the bald-faced hornet (*Dolichovespula maculata*) and of *Vespula* sp. agree with those found by Heinrich (1984). Sphecoid wasps (Apoidea-Spheciformes) also seemed to thermoregulate well above ambient temperature (see Fig. 3.2, green circles). Some species of Sphecidae are known to be active at ambient temperatures as low as 0°C (Evans *et al.*, 1980), and Coelho *et al.* (2007) found that western cicada killers regulated their thoracic temperature by relying primarily on physiological mechanisms. Finally, the only parasitic species of our dataset, *Therion petiolatum* (superfamily Ichneumonoidea) had the second lowest T_{th}-T_a of all species collected (3.7°C; Fig. 3.2, yellow circle), and was generally active during the hotter hours of the summer. We could not find any study on thermoregulation in Ichneumons, but our data suggests that they are poor thermoregulators.

Membrane fatty acid composition is not only linked to body mass and temperature, but it is also believed to be related to longevity through the influence of diet on the peroxidation index (Pamplona *et al.*, 2000). Evidence suggests for example that fatty acids present in the diet exert an influence on the longevity of honeybees, as for example an important addition of either 18:1 or 18:2 to the pollen impairs lifespan (Manning, 2006; Haddad *et al.*, 2007). Here, we found that more ectothermic Hymenopterans had higher PI values in their membranes than more endothermic species (Fig. 3.6D), suggesting that better thermoregulators are less prone to peroxidative damage. However, no significant relationship was found between body mass and PI (Fig. 3.6C), contrary to our results for Euglossine bees (Chapter 2).

Differences in dietary availability could explain the high variability in 18:3 and 18:2 content among species. This variability is partly responsible for the lack of relationship between unsaturation index and body mass or thoracic temperature excess (Fig. 3.6A). For example, bumblebee species studied in the UK were shown to have a diet breadth correlated with flower abundance, and the majority of the pollen collected was from plants of the family *Fabaceae* (Goulson *et al.*, 2008), presumably rich in 18:3 (Manning, 2006). Honeybees are generalists flower visitors and have low 18:3 contents in their thoracic membrane (4%; Fig. 3.4C). It is therefore tentative to suggest that the differences in polyunsaturates among species are due to differences in their diets, but a detailed analysis of each species' regime and pollen lipid composition would be needed to further strengthen this hypothesis. Thus far, evidence suggests that pollen and nectar dietary lipid content has an effect on the thoracic membrane composition and the overall physiology of the bee.

Conclusions

In conclusion, Hymenopterans with differing degrees of thermoregulatory capacity also differ in the fatty acid composition of their thoracic membranes. Species functioning with a low thoracic temperature excess have less 18:1 (MUFA), and more 18:3 and total PUFAs than more endothermic bees and wasps. This relationship follows the predictions of the HVA theory formulated for ectothermic organisms. However, perhaps contrary to this theory, SFA content (16:0 and 18:0) decreases with increasing thorax temperature excess, although the length of the chain could be of important influence.

In agreement with the literature, body mass and thoracic temperature excess were strongly linked in our study, although mass is not the only factor dictating thoracic temperature. Moreover, there were systematic variations of different fatty acids with body mass, in the directions predicted by the "membrane pacemaker" theory of metabolism, although thermoregulation seems to play a more important role in driving membrane composition. As such, when controlling for body mass in our temperature excess-membrane composition relationships, these remained significant in the analysis of residuals. Therefore, it can be concluded that temperature excess and membrane composition are linked, independently from mass. These findings strengthen the idea of membrane composition modulation in the context of homeoviscous adaptation for these singular heterotherms.

There is important variability in polyunsaturated fatty acid content that does not follow the predictions dictated by body mass (18:2 and 18:3) or thoracic temperature (18:2). Diet could play an important role in setting the PUFA content of the membrane, as flower visitation varies between species and so do nectar and pollen lipid composition among plants. A potential impact of these changes in PUFA composition lies in the susceptibility of the membrane to peroxidation, which varies with temperature but not mass among Hymenopterans, potentially influencing lifespan. Further work on diet and changes in membrane composition is needed to assess the influence of fatty acids on Hymenopteran physiology.

Figures and tables

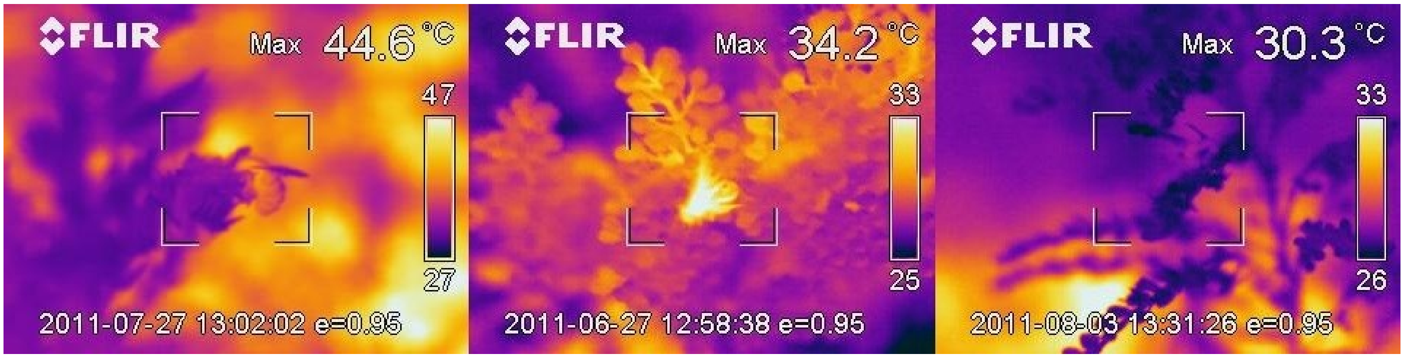

Figure 3.1. Thermograms of different Hymenopteran species collected in the field, showing variation in thoracic temperature: *Bombus bimaculatus* (left), *Andrena* sp. (center), *Polistes* sp. (right). In our experiments, specimens were captured and transferred to a restraining device to measure thoracic surface temperature.

Table 3.1. Number and detailed classification of sampled Hymenopteran specimens (total *n*=130). Some specimens could only be identified to the Subgenus level. Queens (q) and workers (w) are indicated.

Superfamily (Series)	**Family**	**Subfamily**	**Genus**	**Species**	***n***
Apoidea (Anthophila)	Andrenidae	Andreninae	*Andrena (Leucandrena)*	Sp.	4
	Andrenidae		*Andrena (Melandrena)*	Sp.	8
	Andrenidae		*Andrena (Scrapteropsis)*	Sp.	2
	Andrenidae		*Andrena (Trachandrena)*	Sp.	6
	Apidae	Apinae	*Apis*	*mellifera*	4
	Halictidae	Halictinae	*Augochlora*	*pura*	5
	Apidae	Apinae	*Bombus*	*affinis (q)*	3
			Bombus	*bimaculatus (q)*	10
			Bombus	*bimaculatus (w)*	5
			Bombus	*griseocollis (w)*	2
			Bombus	*impatiens (q)*	14
			Bombus	*rufocinctus (q)*	3
			Bombus	*rufocinctus (w)*	2
			Bombus	*ternarius (q)*	5
	Apidae	Nomadinae	*Nomada*	*ruficornis group*	2
	Megachilidae	Megachilinae	*Osmia*	*lignaria*	2
Apoidea (Spheciformes)	Crabronidae	Bembicinae	*Bicyrtes*	*quadrifasciatus*	8
	Sphecidae	Sceliphrinae	*Chalybion*	*californicum*	3
	Sphecidae	Sceliphrinae	*Sceliphron*	*caementarium*	3
Vespoidea	Vespidae	Eumeninae	*Ancistrocerus*	*catskill*	2
		Vespinae	*Dolichovespula*	*maculata*	2
		Eumeninae	*Eumenes*	*fraternus*	3
		Polistinae	*Polistes*	*dominula*	5
			Polistes	*fuscatus*	13
		Vespinae	*Vespula*	*flavopilosa*	4
			Vespula	*maculifrons*	3
Ichneumonoidea	Ichneumonidae	Anomaloninae	*Therion*	*petiolatum*	7

Table 3.2. Sampled Hymenopteran species ranked by mean body mass and with their thoracic temperature excess (±SE) (*n*=130). Queens (q) and workers (w) are indicated.

Genus	Species	*n*	Body mass (g)	T_{th}-T_a (°C)
Augochlora	*pura*	5	0.031 (±0.006)	8.08 (±0.920)
Eumenes	*fraternus*	3	0.035 (±0.007)	6.28 (±0.933)
Andrena (Leucandrena)	Sp.	4	0.037 (±0.006)	8.39 (±1.011)
Ancistrocerus	*catskill*	2	0.039 (±0.003)	9.60 (±1.061)
Therion	*petiolatum*	7	0.042 (±0.008)	3.67 (±0.365)
Andrena (Scrapteropsis)	Sp.	2	0.044 (±0.002)	14.00 (±2.192)
Nomada	*ruficornis group*	3	0.027 (±0.004)	3.60 (±0.283)
Andrena (Trachandrena)	Sp.	6	0.061 (±0.008)	10.62 (±1.071)
Osmia	*lignaria*	2	0.062 (±0.014)	6.33 (±0.028)
Vespula	*flavopilosa*	4	0.068 (±0.003)	13.53 (±1.124)
Bombus	*rufocinctus (w)*	2	0.071 (±0.014)	16.30 (±0.707)
Chalybion	*californicum*	3	0.076 (±0.019)	8.10 (±1.275)
Polistes	*dominula*	5	0.089 (±0.011)	6.30 (±1.116)
Andrena (Melandrena)	Sp.	8	0.090 (±0.003)	13.11 (±1.830)
Apis	*mellifera*	4	0.097 (±0.004)	18.78 (±2.756)
Sceliphron	*caementarium*	3	0.100 (±0.004)	10.12 (±0.777)
Polistes	*fuscatus*	13	0.107 (±0.005)	7.38 (±0.662)
Bicyrtes	*quadrifasciatus*	8	0.113 (±0.013)	12.15 (±0.982)
Bombus	*bimaculatus (w)*	5	0.117 (±0.014)	14.37 (±1.030)
Bombus	*rufocinctus (q)*	3	0.120 (±0.004)	14.17 (±1.568)
Bombus	*griseocollis (w)*	2	0.154 (±0.008)	15.35 (±0.106)
Vespula	*maculifrons*	3	0.169 (±0.003)	16.10 (±3.027)
Bombus	*affinis (q)*	3	0.326 (±0.009)	13.50 (±1.391)
Dolichovespula	*maculata*	2	0.391 (±0.049)	10.52 (±2.675)
Bombus	*bimaculatus (q)*	10	0.393 (±0.015)	20.78 (±1.460)
Bombus	*ternarius (q)*	5	0.424 (±0.017)	14.53 (±0.499)
Bombus	*impatiens (q)*	14	0.550 (±0.016)	17.79 (±0.769)

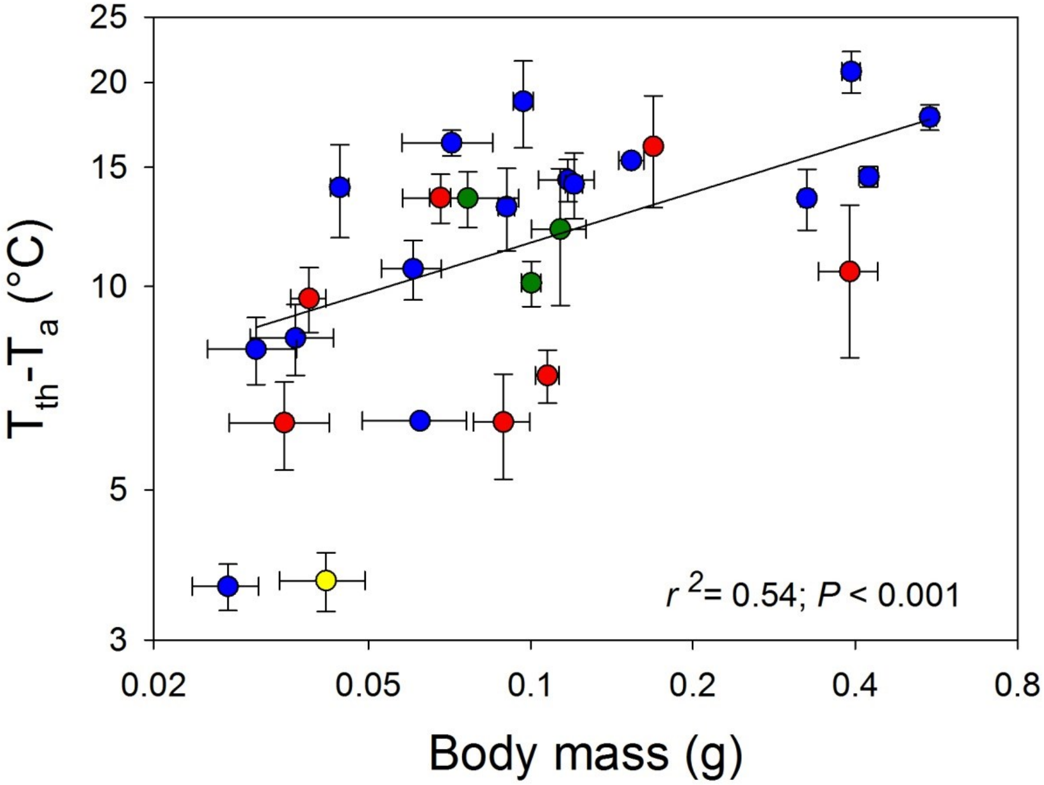

Figure 3.2. Relationship between the thoracic temperature excess and body mass in 27 Hymenopteran species (*n*=2 to 14 individuals). Values are means (± SE). Colors represent the Superfamilies sampled (blue: Apoidea-Anthophila; green: Apoidea-Spheciformes; red: Vespoidea; yellow: Ichneumonoidea).

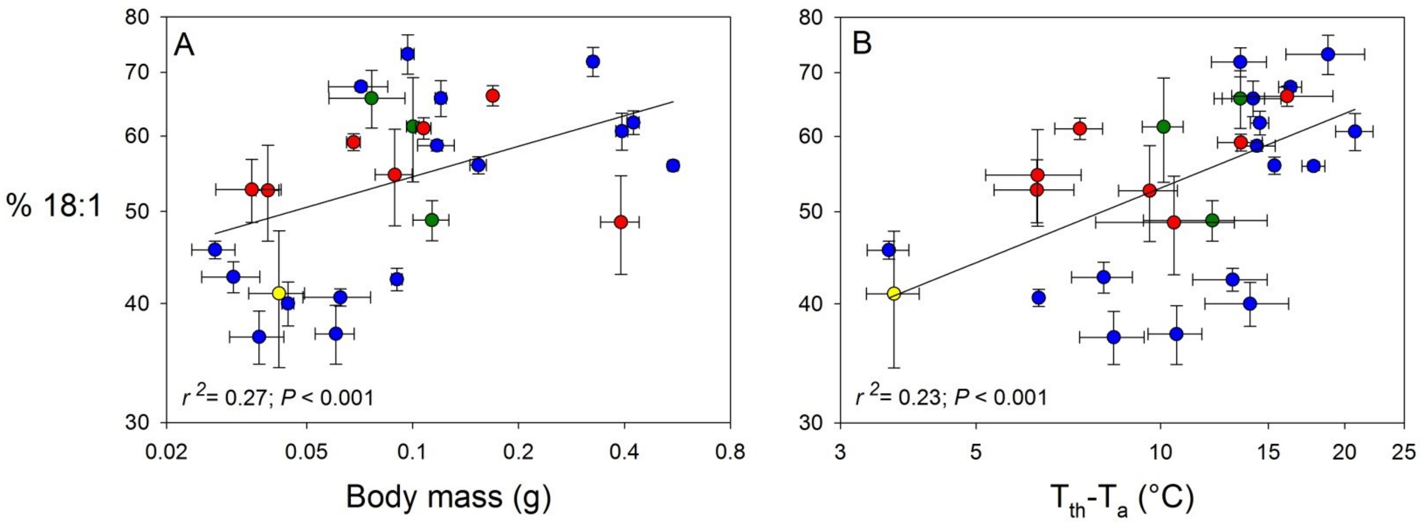

Figure 3.3. Relationship between body mass (A), thoracic temperature excess (B) and the relative abundance of 18:1 (n-9) in the thorax PL of 27 Hymenopteran species (*n*=2 to 14 individuals). Values are means (± SE). Colors represent the Superfamilies studied (blue: Apoidea-Anthophila; green: Apoidea-Spheciformes; red: Vespoidea; yellow: Ichneumonoidea).

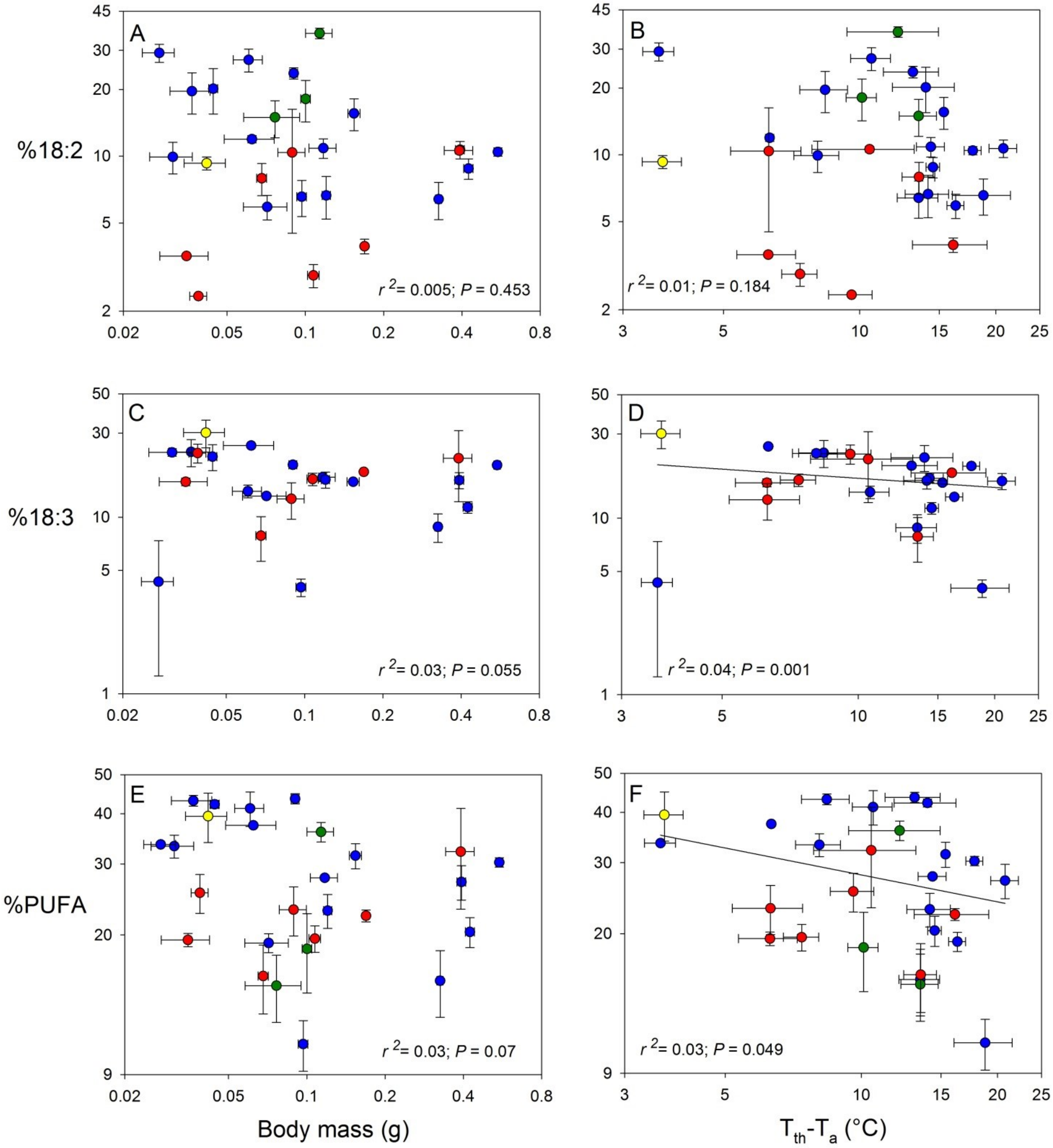

Figure 3.4. Relationship between body mass or thoracic temperature excess and the relative abundance of various polyunsaturated fatty acids: 18:2 (n-6) (A and B), 18:3 (n-3) (C and D) and total PUFA (E and F) in 27 Hymenopteran species (*n*=2 to 14 individuals). Colors represent the Superfamilies studied (blue: Apoidea-Anthophila; green: Apoidea-Spheciformes; red: Vespoidea; yellow: Ichneumonoidea). Spheciformes (green) contained trace amounts of 18:3 (n-3) and were consequently removed of the analysis for this fatty acid.

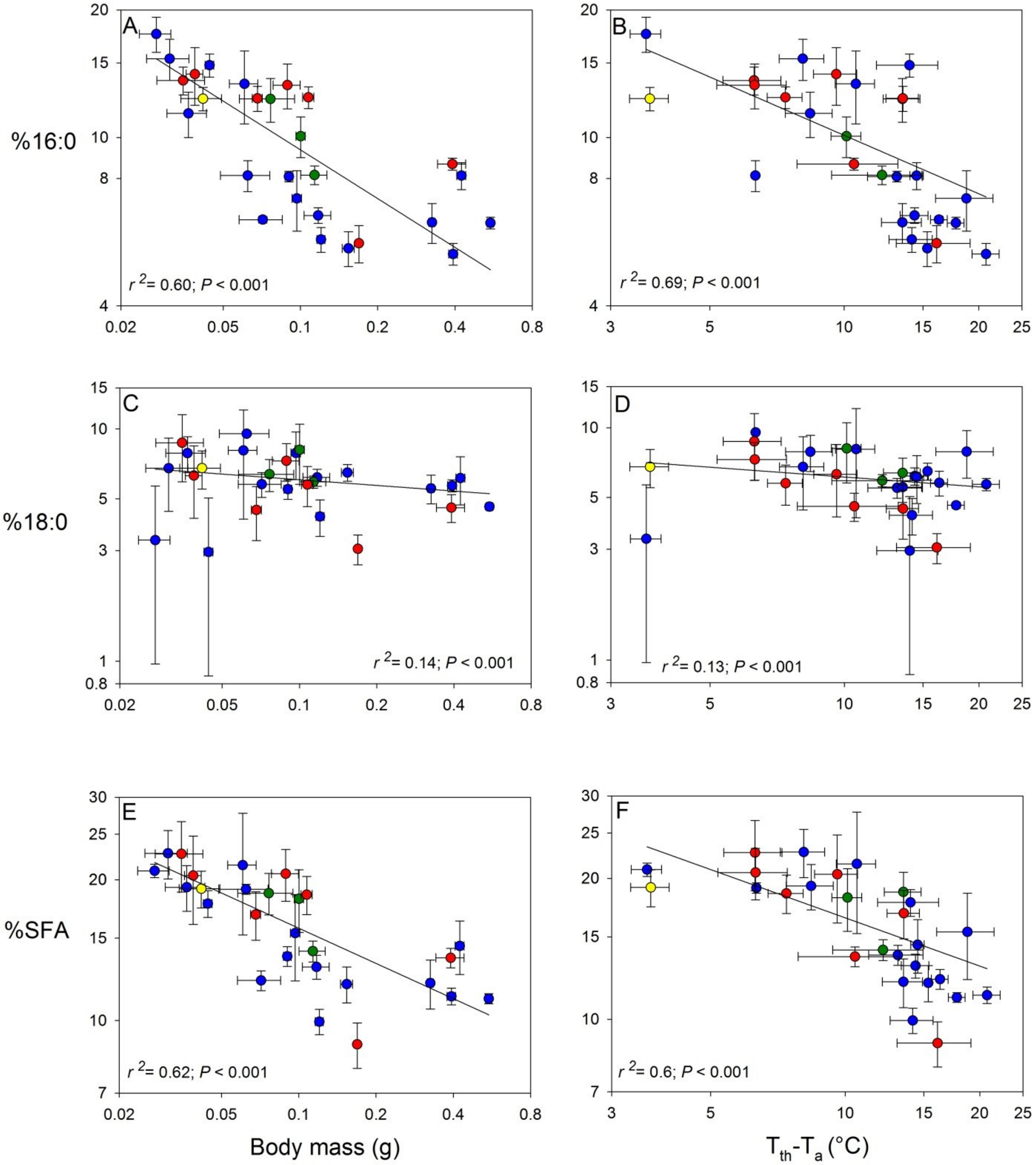

Figure 3.5. Relationship between body mass or thoracic temperature excess and the relative abundance of various saturated fatty acids: 16:0 (A and B), 18:0 (C and D) and total SFA (E and F) in 27 Hymenopteran species (n=2 to 14 individuals). Colors represent the Superfamilies studied (blue: Apoidea-Anthophila; green: Apoidea-Spheciformes; red: Vespoidea; yellow: Ichneumonoidea).

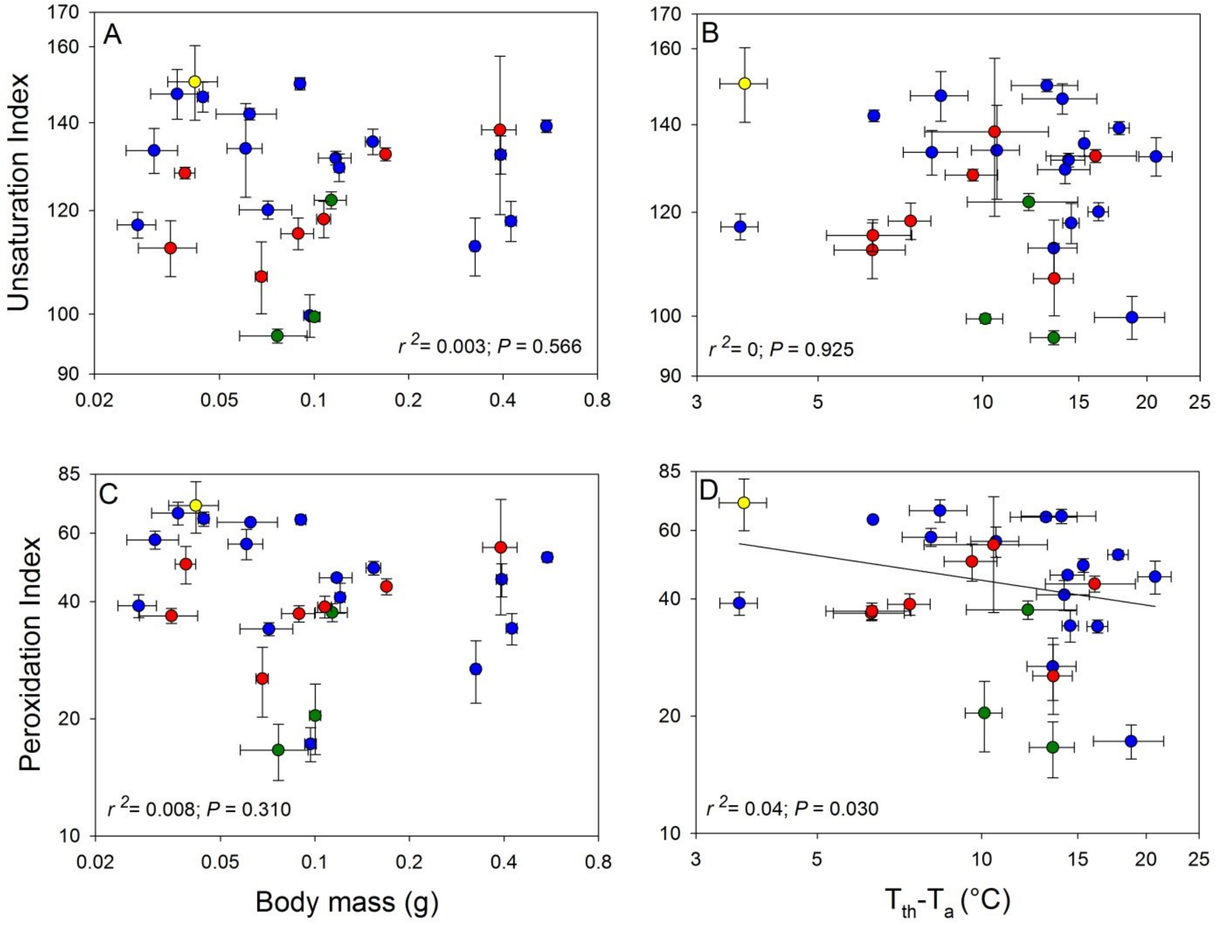

Figure 3.6. Relationship between body mass or thoracic temperature and the unsaturation index (A and B) or peroxidation index (C and D) in 27 Hymenopteran species (n=2 to 14 individuals). Colors represent the Superfamilies studied (blue: Apoidea-Anthophila; green: Apoidea-Spheciformes; red: Vespoidea; yellow: Ichneumonoidea).

Residual analysis to account for the effects of body mass on membrane composition and temperature

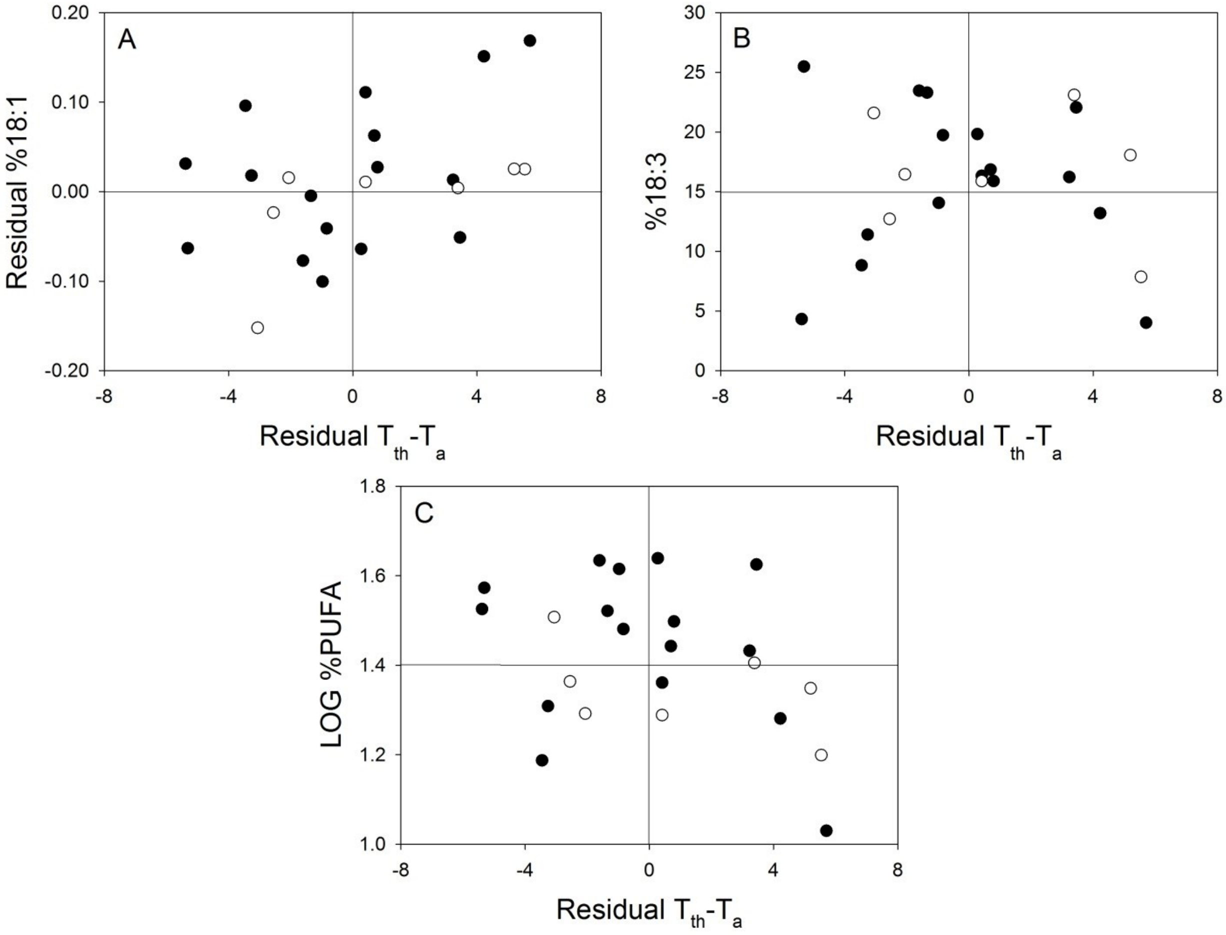

Figure 3.7. Correlations between thorax temperature excess and unsaturated fatty acid residuals obtained from body mass regressions. (A): 18:1 (*r*=0.39; *P*<0.001); (B): %18:3 (n-3) (*r*=0.19; *P*=0.044); (C): LogPUFA (*r*=0.28; *P*=0.004). Values for 23 species of Apoidea-Anthophila (filled circles) and Vespoidea (open circles).

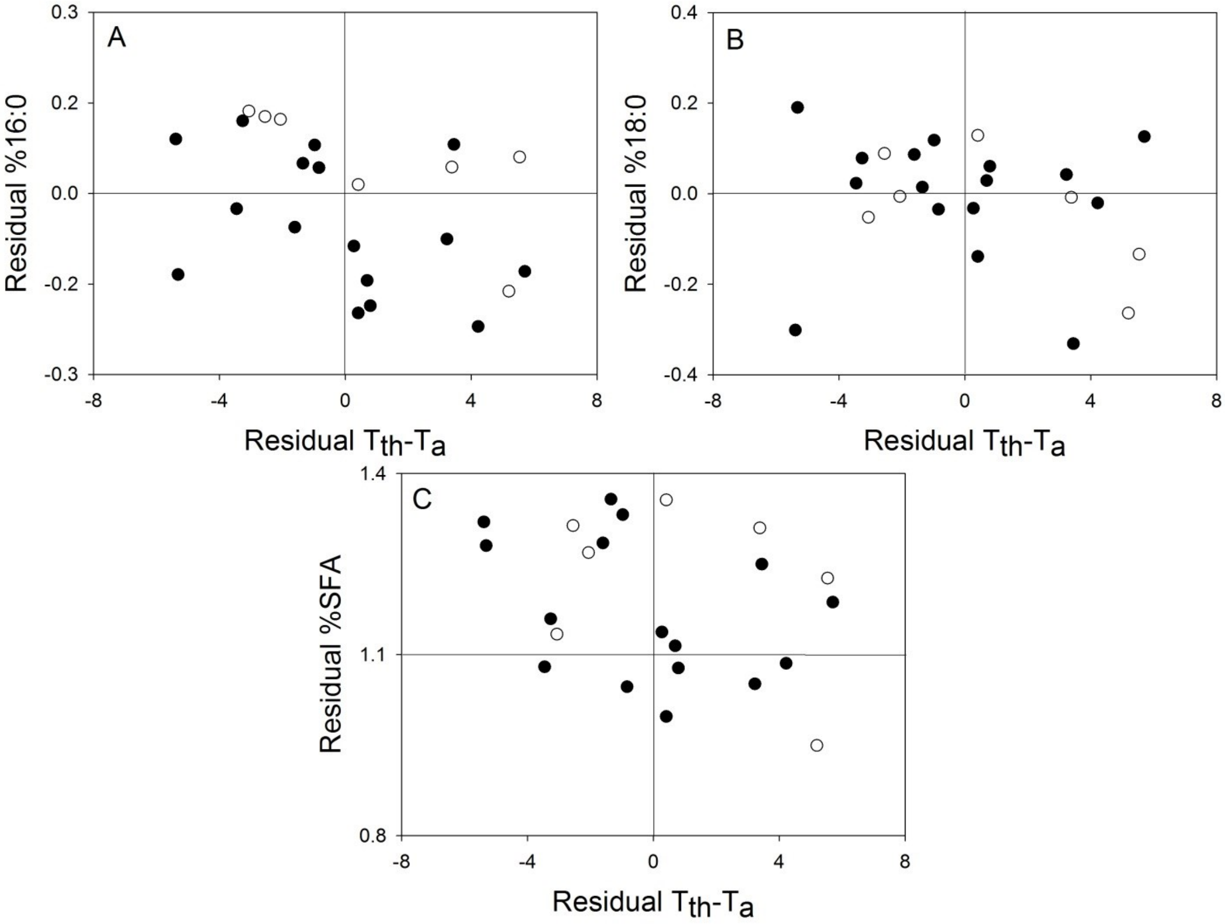

Figure 3.8. Correlations between thorax temperature excess and saturated fatty acid residuals obtained from body mass regressions. (A): 16:0 (r=0.52; P<0.001); (B): 18:0 (r=0.22; P=0.02); (C): SFA (r=0.55; P<0.001). Values for 23 species of Apoidea-Anthophila (filled circles) and Vespoidea (open circles).

CHAPTER FOUR – General discussion

Cell membranes are complex structures and essential to support life, or as Lingwood and Simons (2010) put it, they are: “complicated in composition but precise in purpose: to selectively compartmentalize the constituents of life away from environmental lifelessness.” The discovery that the fatty acid composition of membrane phospholipids in mammals and birds varies systematically with body mass, and that membrane-associated processes are significant determinants of basal metabolic rate has led to the “membrane pacemaker” theory of metabolism. It suggests that modifications in the nature of the membrane bilayer, specifically in the unsaturation of the fatty acid chains, affect rates of molecular activity and are an important determinant of metabolic rate (Hulbert and Else, 1999). The observation of systematic changes in the nature of the lipid bilayer across species living in different thermal environments or following temperature acclimation gave birth to the concept of “homeoviscous adaptation”, where membrane homeostasis is maintained by modifying fatty acid composition, among other strategies (Sinensky, 1974; Hazel, 1995). These two important discoveries have since provided a framework to understand the effects of membrane composition on metabolism, diseases, lifespan, and the influence of diet on these important themes (Hulbert *et al.*, 2005).

Recent studies have raised doubts about the correlated evolution of metabolic rate and membrane composition, central to the “membrane pacemaker” theory (Brzęk *et al.*, 2007; Valencak and Ruf, 2007; Pannorfi *et al.*, 2012). Following the idea that “for many problems there is an animal on which it can be most conveniently studied”, the August Krogh Principle (Krebs, 1975), our research has used species of tropical bees with an important range in size and metabolic rate to refine our understanding of the link between membrane composition, body mass, and metabolic activity. We further investigated diversity in membrane composition in North American bees and wasps that differ in thoracic flight muscle temperature, in the context of homeoviscous adaptation in heterothermic insects. Our results provide support for various aspects of both principles, as well as new insights in the complex world of cell membrane organization.

The scaling of membrane composition with body mass in tropical bees and North American Hymenopterans

Membrane composition in species of tropical and North American bees (Apoidea) was found to be conveniently composed of essentially five fatty acids: two saturates (16:0 and 18:0), one monounsaturate (18:1 n-9) and two polyunsaturates (18:2 n-6 and 18:3 n-3). Fatty acid composition is therefore less diverse than in the skeletal muscle of mammals. In addition to the aforementioned fatty acids, mammals also have significant amounts of 15:1 (n-7), 17:1 (n-7), 20:4 (n-6), 20:5 (n-3), 22:5 (n-3) and 22:6 (n-3) (Couture and Hulbert, 1995). In birds, 18:2 (n-6), 20:4 (n-6) and especially 22:6 (n-3) are among the important polyunsaturates (Hulbert *et al.*, 2002). Alpha-linolenic acid 18:3 (n-3) is found in much lower abundances in mammals and birds than it is in our bee species.

Since the first suggestion by Gudbjarnason *et al.* (1978) of a link between membrane composition and the heart rate of mammals and the finding by Couture and Hulbert (1995) that the acyl composition of various tissues in mammals varies with size, the idea of membranes as "pacemakers" of metabolism has generated numerous subsequent studies on different vertebrate models. Here, in an effort to find evidence for a functional role of membrane composition in setting the pace of metabolic processes, we have found for the first time systematic variations of membrane composition with body mass in an invertebrate model, bees from the superfamily Apoidea (Hymenoptera). Figure 4.1 illustrates our main findings compared with previous work on birds and mammals reviewed in Hulbert (2003). Results reported for mammals and birds show the variation in skeletal muscle 22:6 (n-3), 18:1 (n-9) and total unsaturates (UFA) with body mass (Fig. 4.1A and B). We compare results on vertebrates with tropical and North American Apoidea bees with similar endothermic capacities (May and Casey, 1983) compiled from Chapter 2 and 3 (22 orchid bees and 9 local species) (Fig. 4.1C).

A remarkable feature common to vertebrates and our invertebrate model is that, despite the variations in particular unsaturated fatty acids, the total contribution of unsaturates (UFA, squares; Fig. 4.1) in the membrane stays relatively constant across a wide range of body mass (mammals: r^2=0.002, NS; birds: r^2=0.31, NS; bees: r^2=0.2, P<0.001), despite the small increase found in bees. In mammals, birds and bees, the abundance of MUFA 18:1, oleic acid, increases significantly with mass (mammals: r^2=0.36, P<0.02; birds: r^2=0.66, P<0.05; bees: r^2=0.25, P<0.001). Bigger mass generally means more monounsaturated fatty acids in the membranes, following the predictions of the “membrane pacemaker” theory. The significant decrease in the PUFA 22:6 in mammals and birds (mammals: r^2=0.85, P<0.01; birds: r^2=0.76, P<0.01) counters this increase in oleic acid; while in bees a similar decrease is found in the 18:3 (r^2=0.10, P<0.001), but it does not entirely compensate for the increase in oleic acid. Hence, the two curves do not overlap: bees have a membrane with twice as much 18:1 as birds and mammals and the total UFA increases slightly. Nonetheless, the decrease in the omega-3 (18:3) is consistent with the predictions of the “pacemaker” theory.

Although a high DHA content is associated with an increased activity of membrane-associated processes (Hulbert, 2003; Haggerty *et al.*, 2008), and 18:1 content also affects such processes (Brunaldi *et al.*, 2005), there is no evidence of such an effect of alpha-linolenic acid. Insects could be an ideal model to test this effect, since terrestrial species appear to have low proportions of long-chain PUFAs (and are virtually absent from bee thoraxes), whereas 20-carbon chains PUFAs are more characteristic of aquatic insects (Stanley-Samuelson *et al.*, 1988). The decrease in 18:0 with body mass appears to be also particular to our model: there are no variations in this fatty acid in mammals or birds. However, 16:0 (the other SFA) decreased significantly with size in birds (Hulbert *et al.*, 2002). There is evidence of a role of SFAs in modulating membrane fluidity in insects (Overgaard *et al.*, 2005; Haubert *et al.*, 2008), but whether this class of fatty acids have an effect on membrane-linked processes such as the activity of proteins remains to be investigated.

The number of double bonds per 100 acyl chains, the unsaturation index, decreases significantly with body mass in mammals (r^2=0.74, P<0.01), birds (r^2=0.64, P<0.01) and bees (r^2=0.02, P=0.018). However, the absolute value of unsaturation index is almost two times higher in the smaller mammals than it is in the smallest bees, and the smaller birds have somewhat intermediate values (illustrated in Fig. 4.2). The reason for this two-fold difference is that 22:6 has twice as many double bonds (6) than 18:3, and has more long-chain PUFAs with more than three double bonds (20:4, 20:5 and 22:5). Despite the much greater abundance of 18:1 in bees, given that this PUFA only has one double bond, it is not enough to compensate for the greater weight of 22:6. The difference is mathematical, but it illustrates the fact that for each phospholipid in the membrane, the average number of double bonds likely to be found is twice as high in mammals and birds, than it is in bees. A similar case is found between mammals and reptiles at the same body temperature: the tissue phospholipids in mammals have a much greater unsaturation index than in reptiles, because of the greater abundance of 22:6 and lower 18:1 in mammalian membranes (Hulbert and Else, 1989).

An often overlooked problem that comparative studies face is the lack of independence in the data when comparing various traits. The development of phylogenetic methods to account for the effect of relatedness on trait variations among species helped investigators revisit some of the results linking membrane composition and lifespan or basal metabolic rate (Speakman, 2005; Valencak and Ruf, 2007). However, no study had yet looked at the importance of phylogeny in the context of scaling of membrane composition to body mass. Here, we have shown that even within closely related species of the same superfamily (Apoidea), the presence of a phylogenetic signal (tendency for related species to resemble each other more than by chance) could be detected in some traits (two fatty acids and the sum of saturates), highlighting the contribution of evolutionary history in explaining variation in cellular traits. More importantly, we have shown that when correcting for phylogeny using the independent contrasts method, the relationships between 18:1 and 18:0 remained significant. Thus, using species within the same taxon conveniently eliminates the influence of evolutionary factors on the traits studied. This provides a suitable framework for further testing of the "membrane pacemaker" theory.

Functional implications: metabolic rate and temperature

Although the higher proton permeability (and respiration) of mammalian versus reptile membranes is associated with differences in the fatty acid composition of phospholipids (Brand *et al.*, 1991), the functional link between membrane composition and metabolic rate, central to the "pacemaker" theory, remains unclear (Brzęk *et al.*, 2007; Valencak and Ruf, 2007; Pannorfi *et al.*, 2012). Here, measurements of whole-animal metabolic rate and membrane lipid composition were also done on the same individuals of Euglossine bees, although not included in this thesis. Therefore, the combination of the two variables will be an important follow-up to this study on the relationship between mass and membrane structure. It will provide further claim for or against the "pacemaker" theory of metabolism.

Temperature affects the organization of membranes, and organisms subjected to this environmental variable often need to adjust their membrane structure to ensure functionality. The Homeoviscous Adaptation theory predicts specific changes in phospholipid class and composition (Sinensky, 1974; Hazel, 1995), but these changes vary among ectothermic poikilothermic species. Bees offer an interesting model because of their regional and temporal heterothermy. They can switch from ectothermy to elevated body temperature through endogenous warm-up using their thoracic flight muscle (Stone and Willmer, 1989). Their thermoregulatory capacity varies among species, in a relationship partly, but not entirely linked to their body mass (Stone and Willmer, 1989; Heinrich, 1995; Willmer and Stone, 2004). In our study, we have found that membrane composition varied more closely with the thoracic temperature excess than with body mass. Species of bees with low temperature excess have generally more 18:3 and total PUFA than more endothermic species, and less 18:1 content, even when correcting for body mass. The length of the acyl chain and the level of saturation seems to play a role as well, as poor thermoregulators have more saturates in their membranes. Taken together, these results suggest that membrane composition varies with species' preferred temperature, in accordance with the HVA theory.

Further studies: the importance of diet and implication for lifespan

An extension of the “pacemaker” theory of metabolism links membrane fatty acid composition and longevity of mammals and birds (Hulbert, 2003). More recently, this relationship has also been extended to molluscs with longevities varying between 28 and 507 years (Munro and Blier, 2012). Long-chain polyunsaturates are damaged by reactive oxygen species and are believed to be amplifiers of oxidative stress through the release of reactive carbonyl species. This peroxidation of PUFAs results in increased cellular damage, notably through protein and DNA modification (Hulbert *et al.*, 2007) and promotes cellular aging. The susceptibility of fatty acids to peroxidation can be determined empirically: the value associated is referred to as the peroxidation index (PI), which has been shown to be negatively correlated with longevity. In social insects, the only previous report of such a relationship was found in honeybees (Haddad *et al.*, 2007). Queen honeybees were found to have considerably lower PI than workers, while having a greater longevity. Globally, in bees from the superfamily Apoidea (operating at similar thoracic temperatures), we have found the same decrease with body mass (r^2=0.08, P<0.001; Fig. 4.3). Our results for honeybee workers thorax are within the range found in previous studies (PI = 17.24). However, peroxidation index varies greatly and reaches values similar to those found in the head of honeybees, probably because of the high 18:3 content of some species’ thorax (such as *Andrena Scrapteropsis* Sp., PI = 65.27). Data on longevity for specific bee species is scarce, but long lifespans are generally associated with eusocial species. Many bees have short adult lives of only a few weeks, but some are able to sustain unfavorable seasons in the adult stage (Michener, 2007). More information on the lifespan of the species collected in the present study would be needed to further assess the functional importance of this PI-body mass relationship. We can also note that PI values for bees are up to three times as low as those for the shortest-living mammals and birds. Therefore, it would be of interest to know if the oxidative damage resulting from the peroxidation of 18:3 (the major PUFA in bees) results in important cellular damage potentially affecting lifespan.

Fatty acids are important components of pollen and nectar consumed by bees (Kevan and Baker, 1983). They are also essential for proper development in many insect species, and effects of altered lipid diets on honeybee longevity have been reported (Stanley-Samuelson *et al.*, 1985; Manning, 2006). The fatty acid profiles of certain ichneumonid wasps matches precisely that of the host in which the eggs develop (Stanley-Samuelson *et al.*, 1988). Thus, development appears to be an important step in defining the membrane composition. We have observed important variations in PUFA content (especially 18:2) among Apoidea species of similar body mass. What could be the source of this variation? In mammals and birds, synthesis of SFA and MUFA from non-lipid source is possible, but n-3 and n-6 PUFAs cannot be synthesized *de novo* because they lack the Δ12- and Δ15- desaturases found in plants to do so. Thus, their omega-3 and omega-6 fatty acids must be obtained from the diet. The presence of important quantities of 22:6 (n-3), yet absent from the diet, suggests an important activity of the elongase and desaturase systems in mammals, since the n-3 pathway starts with alpha-linolenic acid and has docosahexaenoic acid (22:6 n-3) as an end-product (Sprecher, 2000). In bees, it is not clear if the Δ12- and Δ15-desaturases are present, since biosynthesis of 18:2 (n-6) has only been found in a few insect orders and there is no evidence of their presence in Hymenoptera (Stanley-Samuelson *et al.*, 1988). Haddad *et al.* (2007) suggest that bees must also obtain n-3 and n-6 fatty acid from the diet. The quasi absence of 22:6 (n-3) in their membranes also suggests lower activities of elongases and desaturases compared to mammals and birds. Moreover, many plants pollinized by bees are known to contain high quantities of 18:2 and 18:3 and their consumption during development could have an important effect on membrane thorax composition and other aspects of their physiology (Manning, 2006). Therefore, more work is needed to assess the relative importance of diet in setting the PUFA content of bee species and its physiological relevance.

Figures

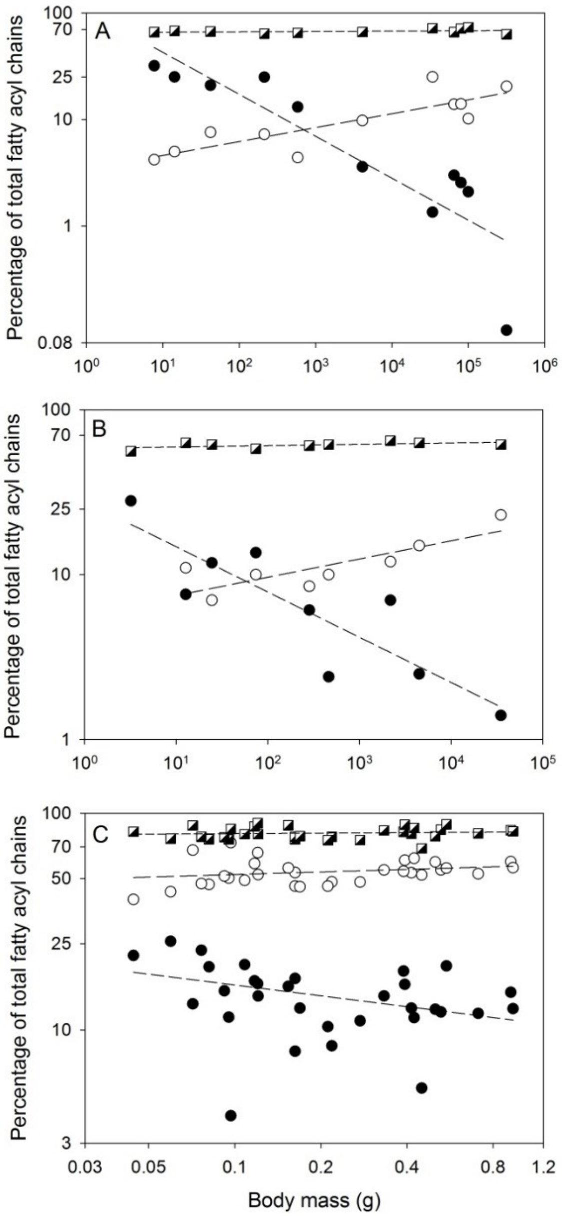

Figure 4.1. The relationship between body mass and acyl composition of membrane phospholipids in mammalian (A) and bird (B) skeletal muscle, and in bees (Apoidea) thoraxes (C). The percentage of total unsaturates (squares; mammals: r^2=0.002, NS; birds: r^2=0.31, NS; bees: r^2=0.2, P<0.001), of the MUFA 18:1 (n-9) (open circles; mammals: r^2=0.36, P<0.02; birds: r^2=0.66, P<0.05; bees: r^2=0.25, P<0.001) and of the PUFA 22:6 or 18:3 (n-3) (filled circles; mammals: r^2=0.85, P<0.01; birds: r^2=0.76, P<0.01; bees: r^2=0.10, P<0.001) are presented. Mammal and bird data are from Hulbert (2003). Apoidea data are from the 22 species of Euglossine (Chapter 2) and 9 species of local bees with evidence of similar endothermic capacity (Chapter 3).

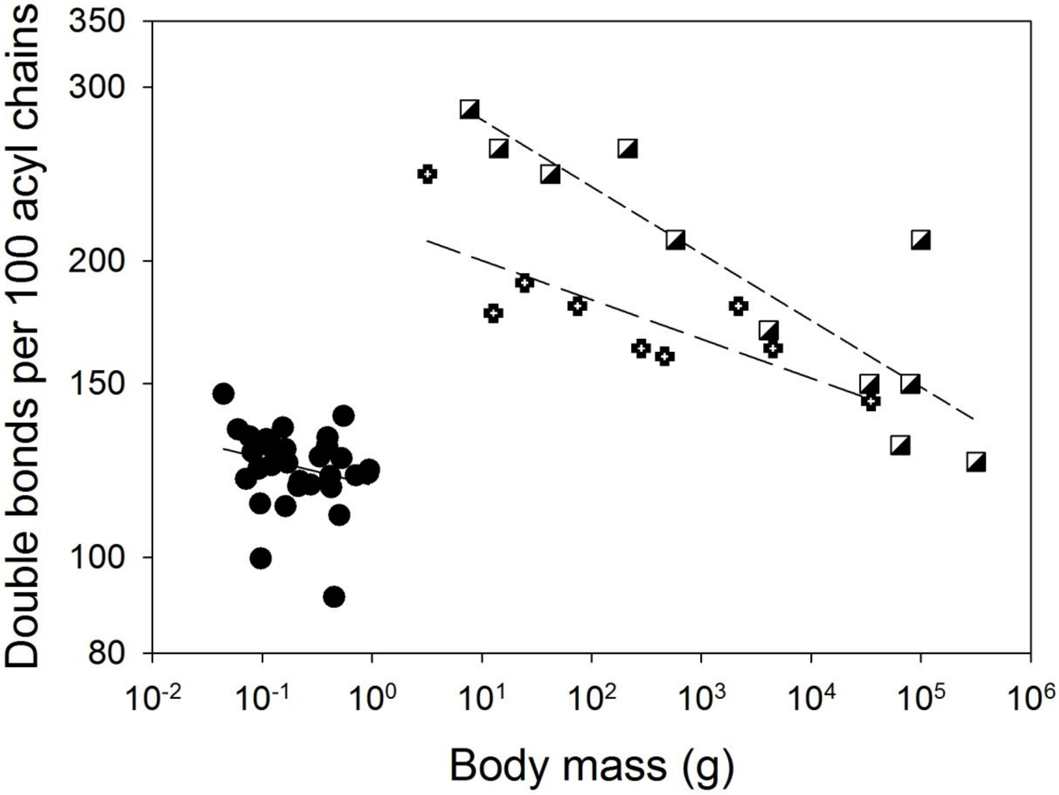

Figure 4.2. The unsaturation index for mammals (squares), birds (crosses) and bees (circles). Regression values are significant for mammals (r^2=0.74, *P*<0.01), birds (r^2=0.64, *P*<0.01) and bees (r^2=0.02, *P*=0.018). Data for mammals and birds are from Hulbert (2003). Apoidea data are from the 22 species of Euglossine (Chapter 2) and 9 species of local bees with evidence of similar endothermic capacity (Chapter 3).

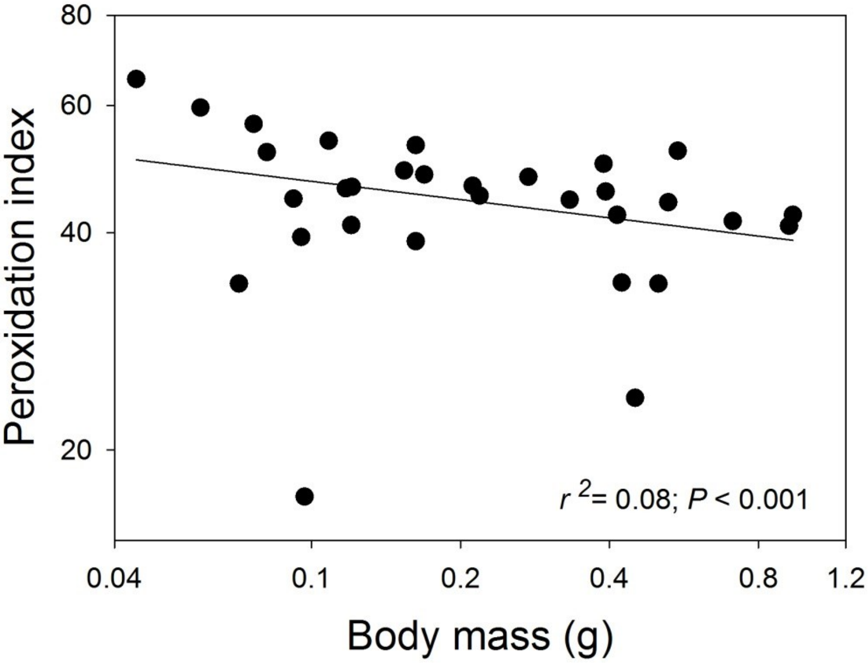

Figure 4.3. The peroxidation index (susceptibility of fatty acids to peroxidative damage) as a function of body mass in 22 species of Euglossine (Chapter 2) and 9 species of local bees with evidence of similar endothermic capacity (Chapter 3) (r^2=0.08, P<0.001).

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