

**Phenotypic plasticity and population-level variation in thermal
physiology of the bumblebee *Bombus impatiens***

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
M.Sc. Degree in the Ottawa-Carleton Institute for Biology

Thèse soumise à la
Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention du diplôme de M.Sc. de
l'Institut pour la Biologie Ottawa-Carleton

Acknowledgements

At first, I would like to thank my thesis supervisor, Dr. Charles Darveau, for his mentorship and guidance which have been fundamental to obtain the best of myself along this thesis. I am also grateful to my committee members, Dr. Gabriel Blouin-Demers and Dr. Jeff Dawson, for their help to refine my protocol and for their constructive criticism.

A special thank you to Dr. Antoine Morin who helped me to understand what a p value and other mysterious statistical terms stand for. Many thanks to Bill Fletcher from ACVS, for taking really good care of my bumblebee colonies after their long trip across Ontario. Thanks to multitasking Dr. Fabien Avaron and Dr. Matthieu Delcourt for their TA supervision.

I would also like to thank all my current and past labmates of the Darveau lab (Deirdre, Dimitri, Enrique, Patrick, Nathalie, Sara) for our brainstorming meetings and their input into my project. Thanks to Chinmay Roy for helping me to stay awake during my long experiment days, and for our language practice: he helped me to improve my English, and I hope he will remember some French words I taught him. I sincerely thank Fannie Billardon, for her constant moral support, her friendship and her efficient fieldwork 24/7.

I am grateful to lots of UofO graduate students, for their encouragement along this journey and their help in my writing: Nora, Julie, Laura, Courtney, Maria, Villie, Daniella, Anna, Machi, Rafael and Hector. I would like to particularly thank Shinjini Pal for her exceptional proofreading skills and motivation.

Thank you to all generous people who helped me during my fieldwork. In particular, I would like to thank The National Capital Commission (NCC), Dustin Drew, Park manager at Lackawanna State Park (Dalton, PA), Chris Dailey, Horticulture Manager at Jacksonville Zoo and Gardens (Jacksonville, FL) and Shane Tippett, Assistant Executive Director for Finance and Operations at Lewis Ginter Botanical Gardens (Richmond, VA), for letting me sample bumblebees on their land. I will never forget the kindness and hospitality of the people in North Carolina, particularly of all the staff working at Airlie Gardens (Wilmington, NC). I notably address my gratitude towards Jim McDaniel and Matt Collogan, respectively Director and Environmental Education Manager at Airlie Gardens, who were extremely welcoming and worked hard to make sure Fannie and I would be comfortable and find a more than decent accommodation to stay safe in stormy weather.

Je souhaite remercier ma famille pour son support, son enthousiasme et son optimisme à toute épreuve. Maman, Denis, Papa, Julie, Nicolas, merci d'avoir toujours soutenu mes projets, même au-delà des mers. Je veux aussi remercier tout particulièrement mes amis. Que je vous ai connus en France, au Québec, au BC ou en Ontario, que vous soyez tout près ou de l'autre côté d'un océan ou d'un continent, vous êtes restés des amis fidèles, sincères et compréhensifs lorsque j'étais trop occupée à travailler sur cette thèse. Merci pour votre patience et vos encouragements. Entre autres, merci à Gen Cool pour son énergie rayonnante, merci à la bande de Vigéens et à la voyageuse Carine-NZ pour votre Chaleur, merci à ma colloc d'enfer Nayelli pour nos longues discussions, merci à Julie Nadeau et Carine Verly pour nos équipes de choc en labo et sur le terrain, merci à ma cohorte de Bacc à l'UQAR pour leur accueil au pays, merci à LnB pour sa motivation contagieuse, et bien-sûr merci à Manue pour son amitié inconditionnelle et éternelle.

This research was also supported by Natural Sciences and Engineering Research Council (NSERC) and by teaching assistantships at UofO. In addition, research expenses were covered by an NSERC Discovery Grant, a Canada Foundation for Innovation Grant and an Ontario Research Fund Grant to Dr. Darveau.

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List of abbreviations

ANOVA : analysis of variance

ANCOVA : analysis of covariance

CTmin : critical thermal minimum

DGE : discontinuous gas exchange

FlightMR : flight metabolic rate

MCA : metabolic cold adaptation

MR : metabolic rate

RMR : resting metabolic rate

RestingMR : resting metabolic rate

SD : standard deviation

SE : standard error

Ucrit : critical sustained speed

WBF : wingbeat frequency

Abstract

Temperature variation affects most biological parameters from the molecular level to community structure and dynamics. Current studies on thermal biology assess how populations vary in response to environmental temperature, which can help determine how populations differentially respond to climate change. To date, temperature fluctuation effects on endothermic poikilotherms such as the common eastern bumblebee (*Bombus impatiens*) are unknown even though bumblebees are the most important natural pollinators in North America. A cold-acclimation experiment with *B. impatiens* colonies revealed that individuals acclimated to 5°C or 10°C at night did not differ in resting metabolic rate, flight metabolic rate, wingbeat frequency, or morphological measurements, compared to the control group. Moreover, an infrared camera showed that all colonies maintained maximum nest temperature consistently above 36.8°C. A latitudinal sampling of flight metabolic rate and morphological measurements of *B. impatiens* from four locations spanning Ontario (N 45°; W 75°) to North Carolina (N 34°; W 77°) indicated no latitudinal trend in the measured variables. This study shows that bumblebees are well equipped to face a wide range of environmental temperatures, both in the short term and long term, and can use a combination of behavioural and physiological mechanisms to regulate body and nest temperatures. These results are reassuring on the direct effects of climate change on bumblebee ecology, but further studies on the indirect effect of temperature variation on North American bumblebees are required to predict future ecosystem dynamics.

Résumé

Les changements de température affectent la plupart des variables biologiques, du niveau moléculaire jusqu'à la structure et la dynamique des populations. Les études en biologie thermique évaluent les réponses des populations face aux températures environnementales, ce qui peut aider ensuite à déterminer les réponses des populations face aux changements climatiques. Jusqu'à maintenant, l'effet de la variation de température sur les endothermes poïkilothermes tels que le bourdon fébrile (*Bombus impatiens*) est inconnu. Pourtant, les bourdons sont les pollinisateurs naturels les plus importants en Amérique du Nord. Des colonies de *B. impatiens* ont été exposées à 5°C ou 10°C durant la nuit pendant au moins trois semaines, alors qu'un groupe témoin était placé à 25°C en permanence. L'expérience n'a révélé aucun effet d'acclimatation au froid sur le taux métabolique de repos, le taux métabolique de vol, la fréquence de battement des ailes et les mesures morphologiques effectuées. Aussi, l'enregistrement vidéo à l'aide d'une caméra infrarouge a montré que toutes les colonies conservaient la température maximale du nid au-dessus de 36.8°C en toutes circonstances. Par ailleurs, le taux métabolique de vol et des caractéristiques morphologiques ont été mesurés sur des bourdons *B. impatiens* provenant de quatre sites d'échantillonnage distribués entre l'Ontario (N 45°; W 75°) et la Caroline du Nord (N 34°; W 77°). Aucune des variables analysées était linéairement corrélée à la latitude. Cette étude montre que les bourdons semblent bien équipés pour affronter une large étendue de températures environnementales, autant sur le court terme que sur le long terme, grâce à la combinaison de mécanismes comportementaux et physiologiques pour réguler leur température corporelle et la température de la colonie. Ces résultats rassurent quant aux effets directs des changements climatiques sur l'écologie des bourdons. Toutefois, afin de modéliser la dynamique future des écosystèmes, il faudra également davantage étudier les effets indirects de la variation de température sur les bourdons d'Amérique du Nord.

INTRODUCTION

Temperature has been considered as one of the most pervasive environmental variables in biological functions. It alters molecular structure (principally lipids and proteins) and chemical reaction velocity (Hochachka and Somero 2002). For example, in ectothermic species exposed to cold temperatures, enzyme-catalyzed reactions are slowed down and biological membranes become more viscous (Cossins and Prosser 1978, Somero 1995). These biochemical alterations influence all physiological functions and are reflected at the whole animal level which ultimately affects the individual's fitness (e.g. through foraging, feeding, escaping predators, and during social interactions). That is why selective pressures lead animals to adopt multiple adaptive strategies, including behavioural, morphological or physiological adjustments, to meet their needs in variable thermal habitats (reviewed in Angiletta et al. 2006).

When behavioural adjustments (e.g. decreased activity, nocturnal vs diurnal activity, basking in the sun, hiding in the shade or in insulated dens) are insufficient for survival in thermally variable environments, animals have evolved other strategies to cope with this variation. Some groups of animals, called stenotherms, can only live within a narrow range of temperature and are usually confined to particular latitudes and/or altitudes, while others, the eurytherms, can cope with various thermal habitats (Somero et al. 1996). Among them, many have developed thermal phenotypic plasticity, the capacity of individuals with the same genotype to exhibit different phenotypes depending on environmental temperature. Adjustment caused by a thermal change within the adult stage of the animal is referred to as thermal acclimation and may be reversible (Seebacher 2005). If temperature change occurs

during developmental stage(s), different and usually irreversible phenotypic modifications may take place (Terblanche and Chown 2006), and this process is sometimes referred to as developmental plasticity. Thus, physiological phenotypic adjustments in response to environmental temperature variation are at the cornerstone of how animals cope with their environment.

Thermal acclimation in ectothermic animals

To date, most studies investigating thermal acclimation have focused on ectothermic poikilothermic species (e.g. reptiles: Seebacher 2005, fish: Lurman et al. 2009, crabs: Tashian 1956, polychaeta: Levinton and Monahan 1983, fruit flies: David et al. 1997, butterflies: Kingsolver 1983, ants: Clusella-Trullas et al. 2010), and have shown a suite of whole-animal responses changing with environmental temperature. As an example, one measure of whole-animal cold thermal tolerance, the critical thermal minimum (CT_{min}), has been extensively studied in ectothermic vertebrates and invertebrates. Literature suggests that populations living in colder environments exhibit lower CT_{min} as predicted from their thermal habitat (Prosser 1955, Fanguie et al. 2006, Terblanche and Chown 2006, Terblanche et al. 2006, McMillan and Sinclair 2011, Darveau et al. in press). In ectothermic invertebrates such as insects, low temperature exposure leads to organism immobilization, a process called chill coma (see Hazell and Bale 2011 for details), and recovery time from chill coma is also associated with population thermal habitat (Fischer et al. 2010, Lachenicht et al. 2010, Ransberry et al. 2011, Schuler et al. 2011). Nonetheless, many studies do not support these trends or show an inconsistent effect of thermal acclimation on cold tolerance capacities in insects. For example, in the butterfly *Lycaena tityrus*, adult acclimation to a colder temperature decreased the recovery time from chill coma only if the individual had

never experienced another chill coma earlier in its life (Zeilstra and Fisher 2005). Additionally, acclimation temperature effects may depend on latitudinal origin. In fact, northern populations of *Drosophila ananassae* showed shorter recovery time from chill coma than populations from lower latitudes, but the reduction in recovery time with decreasing developmental temperature is greater for southern populations than northern ones (Sisodia and Singh 2010). The variation in the effect of acclimation on both CT_{min} and recovery time observed among studies may be explained by the fact that different processes may underlie entry into and recovery from chill coma (Ransberry et al. 2011). Alternatively, different methodological procedures and duration of the experiment to measure critical thermal limits can result in different responses (Terblanche et al. 2007, Chown et al. 2009). Still, it appears that several whole-animal cold temperature tolerance indicators respond to environmental temperature during acclimation, and differ among populations experiencing different thermal habitats.

The impact of reversible thermal acclimation on locomotor performance has been studied in many ectothermic taxa, but equivocal responses have been documented. The effect of acclimation temperature on burst or sprint speed and critical sustained speed, *U*_{crit}, in aquatic and terrestrial ectothermic vertebrates and invertebrates suggests that some species adjust their physiological phenotypes to recover locomotor ability when exposed to low temperatures (Londos and Brooks 1988, Johnson and Bennett 1995, Temple and Johnston 1998, Wilson and Franklin 1999, Marvin 2003, Bailey and Johnston 2005, Glanville and Seebacher 2006). Nevertheless, similar studies performed on other species did not indicate reversible thermal acclimation of locomotor performances (Else and Bennett 1987, Wilson and Franklin 2000, Huang and Tu 2009). Results may also depend on trial temperatures. For

instance, tardigrada (*Macrobotus harmsworthi*) acclimated to 10°C walked faster at 10°C than the group acclimated to 25°C, but the latter walked faster at 25°C than the group acclimated to 10°C (Li and Wang 2005). In insects, *Drosophila melanogaster* populations from California showed the highest maximal walking speed at most temperatures when they developed at 25°C instead of 18°C (Crill et al. 1996), while a population from France performed its highest maximal speed at room temperature when developed at 18°C compared to 25°C or 29°C (Gibert et al. 2001). These studies illustrate the complexity of these effects since the response of populations from various geographical regions can exhibit opposite trends. Nonetheless, Frazier et al. (2008) showed that fruit flies developing in colder temperatures have more success during take-off in cold air due to an increase in wing area and wing length, through developmental plasticity, which highlights the role of developmental plasticity on morphological structures involved in locomotion. Overall, phenotypes associated with locomotion often respond to environmental temperature and display thermal acclimation capacities, but the presence, direction and extent of the responses vary among animals. These apparently ambiguous results are currently being assessed, based on the properties of environmental stressors, suggesting that plastic and non-plastic genotypes would be favoured in different conditions (Gabriel 2005).

The compensatory mechanisms associated with cold temperature exposure are reflected at the whole-animal level through variation in metabolic rate (MR). These observations led to the Metabolic Cold Adaptation (MCA) hypothesis, supported by latitudinal compensation: at a given temperature, some organisms from cooler habitats (or higher latitudes), exhibit higher MR than counterparts from warmer environments (or lower latitudes). This hypothesis has a long history (Krogh 1916) and was applied to many groups

of ectothermic animals, but still remains controversial. For example, Vernberg and Costlow (1966) studied the metabolic response to temperature in fiddler crab (*Uca pugilator*) populations from Florida, North Carolina and Massachusetts. They noticed that the oxygen consumption at cold temperature followed the MCA hypothesis for the adult crabs, but that megalop larvae followed the opposite trend. Yet, Addo-Bediako et al. (2002) performed a meta-analysis including 346 insect species, that provided support for the MCA hypothesis. Nevertheless, the authors pointed out that the effect was highly variable and hemisphere dependent. Along similar lines, Hodkinson (2003) further showed the diversity in patterns emerging when testing the MCA hypothesis in arthropods at small scales, and also highlighted the need to further document geographical and local variation in MR, and that associated with environmental conditions.

Few studies have focused on the intraspecific level (Lardies et al. 2004). Among such studies, many support the MCA hypothesis while others refute it, with no explanation to date except that plasticity induces different costs (Dewitt et al. 1998, Auld et al. 2010) that may be too high, depending on the type of fluctuations displayed by the environmental variable (Gabriel 2005). As an example that followed the MCA hypothesis, grasshoppers (*Aeropedellus clavatus*) from a population living at high elevation consumed more oxygen at all tested temperatures than grasshoppers from a low-altitude population (Hadley and Massion 1985). Another example showed fruit flies (*Drosophila melanogaster*) from colder latitudes in Australia had a higher MR than flies from warmer latitudes when measured at 18°C, which follows the MCA hypothesis; however no difference had been observed between populations found at various latitudes and measured at 25°C (Berrigan and Partridge 1997), implying a different effect of ambient temperature on MR in different populations. Similarly, Lachenicht et al. (2010) showed that crickets (*Acheta domesticus*)

acclimated to 25°C had higher standard MR at 15-25°C than groups acclimated to warmer temperatures; but again, no difference was observed between the acclimated groups when measured at 30-35°C. By contrast, common woodlouse (*Porcellio laevis*) from colder latitudes had lower MR than those from warmer regions (Lardies et al. 2004). In larvae of the Antarctic fly (*Belgica antarctica*), acclimation temperature had no effect on MR, which points out the controversial aspect of MCA (Lee and Baust 1982). We can therefore cast doubt on the generality of MCA, but it remains a useful working hypothesis.

Thermal acclimation in endothermic animals

Acclimation capacities of endothermic homeotherms (birds and mammals) have been studied to a lesser extent. Many studies focused on morphological adjustments, like greater insulation and bigger body size, as the best way to deal with cold temperatures. Many studies confirm seasonal acclimation of thermal conductance of pelage/plumage (Harris et al. 1985, Rogowitz 1990, Maddocks and Geiser 2000, Sheriff et al. 2009), but according to a thermal simulation model, seasonal changes in pelage in small mammals have little effect on mass-specific metabolism (Stuedel et al. 1994). As for Bergmann's rule on body size, it seems well applied among species, and particularly small ones: within a genus, if species were "distinguished as much as possible only by size, the smaller species would all need a warmer climate" (Bergmann 1847, translated by Watt et al. 2010). The rule has been extended within species and is sometimes called James's rule. In a recent review, Pincheira-Donoso (2010) showed that most endothermic homeotherms follow this rule, increasing body size with latitude, amongst and within species, which confirms the importance of morphological adjustments to the environment.

Patterns of physiological thermal acclimation have also been documented in endothermic species, and current studies are starting to investigate the role of adaptive thermoregulation in the context of environmental variation (Boyles et al. 2011). Earlier studies in both birds and mammals showed that many species acclimated to cold temperature exhibited higher resting or basal MR than under control conditions when measured in their thermoneutral zone (reviewed by Chaffee and Roberts 1971). This increase in resting energy expenditure likely reflects the outcome of physiological phenotypic plasticity taking place to cope with the increases in thermoregulatory demands. However, the opposite strategy can be found in many species that decrease or abandon thermoregulation, sometimes by entering torpor or hibernation states. For example, resting metabolic rate (RestingMR) in the southern flying squirrel (*Glaucomys volans*) was at its highest in winter, correlated with a peak of non-shivering thermogenesis (Merritt et al. 2001). The white-tailed jack rabbit (*Lepus townsendii*) also showed a higher RestingMR in the winter (Rogowitz 1990). Moreover, during a laboratory cold-acclimation experiment on six small mammal species (two from subtropics and four from temperate areas), all species displayed an increase of RestingMR, which was significant for both subtropical species and two species originating from temperate climates (Li et al. 2001). By contrast, the deer mouse (*Peromyscus maniculatus gracilis*) consumed less oxygen at 1-2°C in the winter than when measured in the summer at this same temperature (Hart and Heroux 1953). The snowshoe hare (*Lepus americanus*) showed lower RestingMR in winter than in autumn for all given temperatures, but maintained constant body temperature and body mass throughout the seasons (Sheriff et al. 2009). Likewise, winter-acclimatized silvereyes (*Zosterops lateralis*) also had a lower MR at all tested temperatures than birds in the summer, although they had similar body temperature and body mass (Maddocks and Geiser 2000). Finally, acclimation to cold

temperatures did not affect the initial MR of the white-footed mouse (*Peromyscus leucopus noveboracensis*) during exposure to cold temperatures, but it prolonged time before entry into a torpidity state (Hart 1953), which showed a better tolerance for the cold. It is therefore clear that environmental temperature impacts metabolic phenotypes by either increasing oxygen consumption, for instance for increasing thermogenesis, or by reducing metabolic needs with physiological adjustments.

To my knowledge, no intraspecific studies have looked at the effect of thermal acclimation on endothermic poikilotherms. Therefore, in this study, I focus on the effect of cold temperature acclimation on the energetic phenotypes of an endothermic poikilotherm, the bumblebee *Bombus impatiens*. In the first part of the study, I performed an acclimation experiment to assess whole animal response to thermal change. In the second part, I compared metabolic and morphological traits for different populations along a latitudinal gradient.

Study Model

Bumblebees are important natural pollinators in North America for a large number of plants, including economically valuable tomato, blueberry and alfalfa. In contrast to introduced honeybees (*Apis mellifera*), bumblebees are more reliable pollinators since they are faster (Goulson 2010) and forage in cold and rainy weather (Corbet et al. 1993). In the early spring, the queen emerges and starts her colony, which will contain up to 400 workers, before starting to produce males and new queens at the end of the summer. The full developmental time of a bumblebee (egg - larvae - pupae) is four to five weeks and worker longevity varies around four weeks (Goulson 2010). *B. impatiens* colonies are commercially

available in Canada and they are easily maintained in a laboratory with minimum care. This species is abundant, not too difficult to collect in the field, with a distribution overlapping several climate zones from Southern Ontario to Northern Florida.

Like several flying insects, bumblebees are endothermic poikilotherms, a feature which allows them to quickly reach high thoracic temperature in order to fly in various climates, and to regulate the temperature of the nest to keep their brood at a high temperature during development. Bumblebees master temperature better than many other animals (Heinrich 1993), thermoregulating only when necessary. Despite their small size, which leads to high rates of heat loss, they regulate their body temperature above 37°C, even when flying at near freezing temperatures (Heinrich 1993). High thoracic temperature is required before take-off, and flight is essential to bumblebee ecology for nectar foraging, pollen foraging and reproduction. The same muscles are activated for both thermoregulation and for flight, showing the ecological importance of the thoracic muscular system in this species (Heinrich 1993). The only fuels bumblebees use are carbohydrates (Heinrich 1979), keeping a respiratory quotient of 1 (i.e. they release as much CO₂ as they consume O₂) which is convenient when measuring MR.

Flying bumblebees keep warm mostly through the heat produced by flight muscle metabolism, and they maintain a near constant thoracic temperature at all ambient temperatures (Heinrich 1993). In many bee species, higher FlightMR has been reported at lower air temperatures (Harrison et al. 1996, Roberts et al. 1998, Borrell and Medeiros 2004), which was associated with active thermoregulation in flight, but has not been observed in all species (Roberts and Harrison 1998, Harrison and Fewel 2002). Similarly, a negative relationship between wingbeat frequency (WBF) and environmental temperature has been reported in many studies on bees (Roberts et al. 1998, Borrell and Medeiros 2004),

including bumblebees (Roberts and Harrison 1998), although this is not true for all species (see Joos et al. 1991). Both observations led researchers to suggest that many bee species appear to actively thermoregulate in flight to maintain high thoracic temperature at low ambient temperature, but to date, no study has explored the effect of environmental temperature on both FlightMR and WBF in the same bumblebee species (Roberts and Harrison 1998).

Bumblebees have a spectrum of thermoregulatory mechanisms that are used during various activities. Before take-off at low temperature, between two foraging flights, and in the nest to incubate broods, heat can be produced by shivering and non-shivering thermogenesis. The former mechanism uses the powerful antagonist flight muscles that are contracted simultaneously. The dorsoventral and the dorsal longitudinal muscles are linked up to the wings through a small muscle, called the pleurosternal muscle, that disconnects the wings from the shivering muscles when it is relaxed (Heinrich 1979), thus allowing heat production without wing movement. During shivering thermogenesis, the flight muscles are contracting at the same pace as neural stimulation (Heinrich 1995), while during flight, these same muscles become asynchronous, where a single action potential initiate a series of contractions maintained by stretch activation (see Esch and Goller 1991). Newsholme et al. (1972) and Clark et al. (1973) suggested that bumblebees may also use non-shivering thermogenesis when they are resting. “Futile cycles” between fructose-6-phosphate and fructose-1,6-biphosphate would generate heat in flight muscles without contraction, but this thermoregulatory mechanism is still debated (Esch and Goller 1991, Staples et al. 2004). If futile cycles have a role in bumblebee thermoregulation, they would probably be more important in small bees, since they cool down faster than larger ones (Staples et al. 2004), and in the queen before the first brood of workers hatches and helps her heat the nest.

Objectives

The aim of this study was to determine whether cold-acclimation induces plasticity in energetic phenotypes in an endothermic poikilotherm, both at rest and during locomotion. In both ectothermic poikilotherms and endothermic homeotherms, RestingMR has been shown to increase when animals are acclimated to cold temperatures, due to increased costs associated with compensatory mechanisms or increased costs of thermoregulation. It is therefore predicted that bumblebee RestingMR will increase when acclimated to cold temperatures. In addition, thermal acclimation can impact locomotor performance in ectothermic poikilotherms as well as induce morphological changes associated with locomotion. Moreover, endothermic poikilotherms such as bees thermoregulate in flight by increasing wingbeat frequency and increasing metabolic heat production, thus metabolic rate. It is therefore predicted that following cold acclimation, bumblebees would exhibit greater flight wingbeat frequency and metabolic rate, but also that the proportion of wing size to body mass would increase following development in colder conditions.

MATERIAL AND METHODS

Acclimation Experiment

Experimental design

The temperature acclimation experiment took place from March to July 2010. Young colonies of approximately 80 *B. impatiens* workers were shipped in their housing box (29x23x13 cm) by a commercial supplier (Biobest Canada Ltd., Leamington, Ontario, Canada), and received at the University of Ottawa (Ontario, Canada) within 24 to 48 hours. One new colony per week was ordered, for nine weeks. When delivered, each colony was assigned to either the control or one of the experimental incubators (VWR Low temperature diurnal illumination 2015) and maintained in its housing box with a transparent lid for the rest of the experiment. The three incubators were programmed for a 12h:12h light:dark cycle, with night taking place from 8:00 to 20:00. The control incubator was programmed at 25°C day and night (25//25). The treatment incubators were also adjusted to 25°C during daylight, but one was placed at 5°C (25//5) and the other at 10°C (25//10) during the night. Cycling temperatures were chosen as they mimic environmental fluctuations and have been shown to induce phenotypic plasticity in other species (e.g. Pétavy et al. 2004, Niehaus et al. 2012). All colonies were fed *ad libitum* with unlyophilised pollen balls and Biogluc (Biobest Canada Ltd., Leamington, Ontario, Canada) or a sucrose solution (50% w/v).

Measurements

Each type of individual measurement described below was performed four times for each colony. Initial measurements (pre-acclimation state) were performed on adult workers

within the first three days of arrival of the colony. They were marked on the thorax with a water-based opaque paint marker at the end of the series of measurements, and another 30 to 50 adult workers were marked on the third day, for a total of 72 to 92 marked adult bees in the colony. Then, three weeks later (post-acclimation state), the same measurements were performed on this marked cohort. All these tagged bumblebees formed the “first cohort experiment” and represent the adult acclimation experiment. During the sixth week, newly emerged adults that had developed in the experimental conditions were marked, while their silver hair and curved wings differentiated them from mature adults. A different paint colour was used for each day of the week to record the age of the bee. Measurements were performed on this “second cohort” 7 ± 1 days after emergence (pre-acclimation state), and again, two weeks later (post-acclimation state) (see summary in Figure 1). For this 2nd cohort acclimated both during developmental stages and during their adult phase, pre and post-acclimation terms refer to the adult acclimation state. Only two colonies of the 25//10 group and two colonies of the 25//25 group, and none of the 25//5 group, were still producing workers at this point of the experiment. Thus, results of workers that developed in the acclimation conditions are presented only for 25//10 and 25//25 groups.

Flight Metabolic Rate (FlightMR)

Individual bees were captured from the hive and kept in transparent 50 mL tubes until measurements. Holes were punctured on top of the tubes to help aeration, and cotton soaked with sucrose solution was placed at the bottom of the tube in order to standardize hunger levels for all bees. FlightMR measurements on the first cohort (n=627) were performed at three flight trial temperatures (15°C, 25°C and 35°C), while measurements performed on the second cohort (n=73) were performed at 25°C only. To keep a constant

temperature, the respirometry chamber was placed into a temperature-controlled portable cabinet (PTC-1, Sable Systems International, Las Vegas, Nevada; SSI) linked to a temperature-controller (Pelt-5, SSI). Despite temperature variation in the cabinet due to the opening of the lid, target temperature $\pm 1.5^{\circ}\text{C}$ was reached during measurements. Most FlightMR were measured during the day between 8:00 and 20:00, although a few were collected between 6:00 and 8:00.

Individual bees flew in a respirometry chamber consisting of a 1 L glass bottle with a side-arm at the base. Considering that bumblebees exhibit a respiratory quotient equal to 1, showing an exclusive use of carbohydrates, as documented in other bees (Suarez et al. 2005), FlightMR was measured by CO_2 production. Ambient air was drawn, dried with Dessicant Media (PermaPure), and pushed through a flow controller and a flow meter, before entering the respirometry chamber from the bottom side-arm and flowing out through the bottle neck and into a gas analyzer at $1000 \text{ mL}\cdot\text{min}^{-1}$ (FoxBox, SSI). A CO_2 baseline measurement was recorded before and after the presence of the bee in the chamber. CO_2 production rates were recorded and analyzed using the data acquisition and analysis software Expedata (SSI). CO_2 calibration was performed every morning with nitrogen gas and certified 428 ppm CO_2 span gas. Metabolic rate was calculated by measuring CO_2 production from a 1-min steady-state-flight; CO_2 production rate was calculated by subtracting the baseline CO_2 value (CO_2 expressed in %) divided by 100, and multiplied by the flow rate in $\text{mL}\cdot\text{min}^{-1}$. The value obtained was multiplied by 60 to express metabolic rate in $\text{mL CO}_2\cdot\text{h}^{-1}$. Flight quality was described according to six different categories as follows:

Type1: excellent controlled flight with folded legs, usually away from bottle wall, flying by itself, no shaking needed

Type2: good flight with or without folded legs, lands sometimes, no or little shaking needed

Type3: intermittent good flight but lands often

Type4: walking on walls with sparse use of wings to fly

Type5: mostly walking (data not considered)

Type6: mostly still (data not considered)

Preliminary analyses showed no effect of flight types on MR estimates and were therefore not included in further analyses. Furthermore, no effect of the colony on FlightMR was detected, except for the pre-acclimated control group flying at 35°C ($F_{2,32}=4.73$, $p=0.016$ with a 1-way ANCOVA and body mass as covariate). Therefore, bees from different colonies acclimated to the same temperature were pooled for statistical analyses.

Wingbeat Frequency (WBF)

When bees were flying, an optical detector (Moore Scientific) was placed at the bottom of the temperature-controlled cabinet. Sampling bursts of 0.19 s with 44100 samples per second were recorded by T-Rex 2.0 Transient Waveform Recorder software (Moore Scientific) through the sound card of the computer when the bee flew between a flashlight and the detector. For each individual, the average of the first ten measures of WBF was calculated. Due to large amount of data, subsampling with only Type1, Type2 and Type3 flight measurements were sufficient for WBF analysis of first cohort acclimation experiment (n=423).

For each acclimation temperature, in both acclimation state and three flight trial temperatures, no effect of the colony on WBF was observed, except for the pre-acclimated

25//5 group flying at 35°C ($F_{2,16}=5.85$, $p=0.012$ for the colony*body mass interaction term). Since only 1 out of 18 groups showed this colony effect, including a control group, all bees acclimated to the same temperature were pooled for statistical analysis.

Resting Metabolic Rate (RestingMR)

Bees that flew at 25°C were transferred into transparent 50 mL tubes containing sucrose solution, and then were placed into small microrespirometry chambers (SSI) in a dark, quiet room, maintained at 25°C. A pump coupled to a Flowbar-8-Multiplexor was used to push ambient air flowing at 40 to 100 mL·min⁻¹ through seven respirometry chambers and a baseline chamber in parallel. The CO₂ content difference between one of the eight chambers and an internal reference chamber was recorded (CO₂ LiCor 7000 differential CO₂/H₂O analyzer). The measurement cycle started with CO₂ rate from the baseline chamber for 10min, then CO₂ rate from the first chamber for at least 45min (up to two hours if fewer individuals were measured), and going back to CO₂ rate from the baseline chamber before switching to the second chamber, and so forth until all seven bees' RestingMR had been recorded with Expedata data-acquisition software (SSI). Analyses were performed only on individuals showing discontinuous gas exchange (DGE), which was used as an indication of a resting state (Figure 2). The integration of CO₂ production over the highest exact number of cycles (from the beginning of a cycle (just before the peak of the open phase) to the end of a cycle (just before the peak)) was calculated with Expedata software (SSI). For each acclimation temperature, no effect of the colony on RestingMR was observed. Therefore, all bees acclimated to the same temperature were pooled for statistical analysis. Sample size for each acclimation group, before and after acclimation, varied from 51 to 88 workers for the first cohort experiment, and from 14 to 23 workers during the second cohort experiment.

Morphometric Measurements

After flight trials, bees were placed in a refrigerator (4°C) for a few minutes in order to immobilize them and facilitate their handling. Bees were weighed on an analytical scale (Mettler Toledo Excellence XS) to the nearest 0.01 mg.

At the end of the experiment (after nine weeks of acclimation), workers were randomly collected from the colony and frozen at -80°C. Approximately two months later, bees were dissected and their wings were pasted on white sheets of paper. Wing area and maximum length of the left forewing and hindwing were measured on twelve bumblebees per colony, using digital images captured with a calibrated dissecting microscope (Zeiss Discovery V8) and analyzed using Axiovision Release 4.7.1 software. Furthermore, body mass of these workers was measured on the analytical scale. Finally, average wing loading was calculated as the body mass divided by wing area, and expressed in $\text{g}\cdot\text{cm}^{-2}$.

Maximum Nest Temperature (T_{nest})

During the first week of acclimation and three weeks later (pre and post-acclimation states), an infrared camera (ThermaCam EX300) was placed above the nest in the incubator, for approximately 24 h. The maximum temperature of the entire nest was measured using an emissivity of 0.96. It was displayed on the camera screen and recorded with a computer using a video capture interface and software (Pinnacle). Videos were analyzed to obtain the maximum nest temperature (T_{nest}) every 30 min for each colony. Most videos lasted more than 20 h, from 16:00 to 12:00. Data were pooled into six time periods of four hours where three time periods occurred at night (20:00-24:00, 24:00-4:00, 4:00-8:00) and three time periods took place during daytime (8:00-12:00, 12:00-16:00, 16:00-20:00). An average T_{nest}

was calculated for each colony for each time period and statistical analyses were performed on these averages as repeated-measures before and after acclimation.

Field Sampling

Study sites and animal collection

Data collection was conducted in the field in August and September 2010. Five sites in five different climate zones (but along similar longitude) were visited:

- Site 1: Mer Bleue in Ottawa, Ontario (N 45°23 545; W 75°33 072),
- Site 2: Lake Lackawanna in Scranton, Pennsylvania (N 41°33 840; W 75°42 110),
- Site 3: Lewis Ginter Botanical Gardens in Richmond, Virginia (N 37°37 199; W 77°28 168),
- Site 4: Airlie Gardens in Wilmington, North Carolina (N 34°12 902; W 77°49 799), and
- Site 5: Jacksonville Zoo and Gardens in Jacksonville, Florida (N 30°40 133; W 81°64 509).

The most southern site, in Jacksonville, was not considered in the analyses because too few *B. impatiens* were found. In the two most northern sites, bees were caught with insect nets in large fields of wildflowers. At the two other sites, sampling was carried out in Botanical Gardens since wildflowers were sparse and *B. impatiens* was hard to find in natural spaces.

Field data measurements

Flight metabolic rates at 25°C and 35°C were measured in the field with the same equipment as FlightMR measurements during the acclimation experiment. Calibration of the FoxBox was performed prior to a series of measurements using soda lime and certified 397 ppm CO₂ span gas. Bees were maintained in a dark cooler-type bag, each in a 50 mL

transparent tube in presence of sucrose solution until the night, at which point the RestingMR measurements were performed. Since air temperature varied greatly throughout the night and between sites, and the RestingMR set up could not be reconfigured into the temperature controlled cabinet, RestingMR data were not analyzed. Workers were then maintained in liquid nitrogen throughout the road travel, and subsequently stored in a freezer at -80°C . Body mass and wings measurements were performed at the University of Ottawa as described previously for the acclimation experiment.

Statistical analyses

All statistical analyses were performed with Systat 12 and Systat 13 and p values of less than 0.05 were considered significant. A repeated-measures ANOVA was used to evaluate the effect of acclimation temperature and time of the day on the difference between pre and post-acclimation T_{nest} . Two subsequent ANOVA assessed the effect of acclimation temperature and time period on pre-acclimation and post-acclimation T_{nest} separately. ANCOVA and ANOVA tests were used to evaluate the effect of acclimation temperature, acclimation state, flight trial temperature, and body mass on the different dependent variables (FlightMR, WBF, RestingMR, and morphological measurements) for the acclimation experiment. ANCOVA and ANOVA were followed by linear regressions or by post-hoc tests with Bonferroni adjustments for multiple comparisons; given that Bonferroni corrections are conservative, in some cases, non-significant values were further assessed using Tukey tests. Dependent variables obtained during fieldwork (FlightMR, WBF and morphological measurements) were analyzed in the same way to assess the influence of site, flight trial temperature, and body mass on these variables. Normality and homoscedasticity

assumptions for ANOVA and ANCOVA were tested with Kolmogorov-Smirnov and Levene tests, respectively. For some analyses with large sample sizes, visual assessments of normality and homoscedasticity of residuals were performed. For the analysis of RestingMR of the first cohort, both RestingMR and body mass variables were log-transformed to normalize the residuals. Moreover, for this variable an unexplained outlier has been identified by the statistical software (a 230.9 mg bee measured after acclimation 25//10 with a RestingMR of $1.12 \text{ mL CO}_2 \cdot \text{h}^{-1}$, or $4.86 \text{ mL CO}_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1}$) but was not affecting the results; it has been kept in the statistical dataset but removed from figures 5 and 6 for visual presentation. All values are presented as mean \pm SE, except in the Figure 7 showing mean \pm SD. Partial R^2 represents the percentage of variation of the dependent variable explained by the variation of the independent variable in question (see summary of results in Table 1).

RESULTS

Acclimation Experiment

First Cohort Acclimation Experiment

Morphological Measurements

A 1-way ANOVA showed no significant effect of acclimation temperature on body mass after nine weeks of acclimation ($F_{2,104}=1.579$, $p=0.211$, $R^2=0.029$). Similarly, ANCOVAs showed no effect of acclimation temperature on *B. impatiens* wing area (mm^2) and wing loading ($\text{g}\cdot\text{cm}^{-2}$). Body mass explained most of the variation in these variables (respectively $F_{1,105}=1194.98$, $p<0.0001$, $R^2=0.919$ and $F_{1,105}=143.43$, $p<0.0001$, $R^2=0.573$).

Flight Metabolic Rate

A 2-way ANOVA on body mass of bumblebees used for flight metabolic rate (FlightMR) analysis showed that acclimation temperature had no effect on body mass, but that body mass varied with acclimation state. Mean pre-acclimation body mass was 188.78 mg with individuals varying from 83.3 mg to 343.80 mg, while mean post-acclimation body mass was 214.17 mg with individuals varying from 89.8 mg to 391.50 mg.

The effect of acclimation temperature, acclimation state, and flight trial temperature on FlightMR of worker bumblebees is shown in Figure 3A. An ANCOVA was performed using these three factors and body mass as a covariate ($R^2=0.565$). Body mass explained 12.7% of FlightMR variation ($F_{1,618}=179.75$, $p<0.0001$). FlightMR was higher at 15°C (15.21 ± 0.25 mL $\text{CO}_2\cdot\text{h}^{-1}$, $n=173$), followed by 25°C (12.90 ± 0.22 mL $\text{CO}_2\cdot\text{h}^{-1}$, $n=226$) which was higher than 35°C (8.94 ± 0.20 mL $\text{CO}_2\cdot\text{h}^{-1}$, $n=228$). The effect of flight trial

temperature was highly significant and explained most of the variability in FlightMR ($F_{2,618}=241.87$, $p<0.0001$, partial $R^2=0.341$). FlightMR significantly varied among the three flight trial temperatures for all acclimation temperatures, in both pre and post-acclimation states ($p\leq 0.001$ for all comparisons except for bees after acclimation at 25//10°C flying at 15°C and 25°C ($p=0.069$; $p=0.044$ with Tukey test), and between 15°C and 25°C for bees before acclimation to 25//5°C ($p=0.171$; $p=0.096$ with Tukey test). The acclimation state had a significant effect on FlightMR ($F_{1,618}=89.62$, $p<0.0001$, partial $R^2=0.064$), where post-acclimation FlightMR was lower for all three acclimation temperatures at all three flight trial temperatures ($p\leq 0.0001$), except at 15°C for bees acclimated to 25//5°C ($p=1.000$; $p=0.615$ with Tukey test), at 25°C for bees acclimated at 25//10°C ($p=0.141$; $p=0.082$ with Tukey test) and at 35°C for bees acclimated at 25//25°C ($p=0.204$; $p=0.111$ with Tukey test). The interaction of body mass and acclimation temperature was significant ($F_{2,618}=5.346$, $p=0.005$, partial $R^2=0.008$) and reflects the fact that body mass had a significant effect on FlightMR for some groups but not for others. The effect of the acclimation temperature factor was also significant ($F_{2,618}=7.495$, $p=0.001$, partial $R^2=0.011$). In fact, of 18 possible pairwise comparisons, only two were significantly different: pre-acclimation, FlightMR at 15°C was lower for bees acclimated at 25//5 than for bees from the control group 25//25 ($p=0.013$), and similarly, post-acclimated 25//5 bees showed a lower FlightMR at 35°C than 25//25 bees ($p=0.008$).

Wingbeat Frequency

Wingbeat frequency (WBF) was analyzed using an ANCOVA ($R^2=0.470$) including the effect of acclimation temperature, acclimation state, flight trial temperature, and body mass as a covariate. Acclimation temperature and flight trial temperature had no statistically

significant effect on WBF (Figure 3B). Most of the variation in *B. impatiens* WBF was explained by body mass ($F_{1,419}=265.81, p<0.0001, \text{partial } R^2=0.336$). Pre or post acclimation state ($F_{1,419}=27.36, p<0.0001, \text{partial } R^2=0.035$), and the interaction between body mass and state ($F_{1,419}=17.61, p<0.0001, \text{partial } R^2=0.022$) also showed a significant effect on WBF, but with much lower explained variance (Figure 3B). A post-hoc test showed no significant difference between pre and post-acclimated WBF for all three acclimation temperatures at all three flight trial temperatures. However, a linear regression describing the effect of the acclimation state and body mass on WBF of bumblebees showed a different slope between pre and post acclimation states for all three acclimation temperatures (Figure 4). For 25//25, $\text{WBF} = -0.234\text{mass} + 241.53$ before acclimation and $\text{WBF} = -0.147\text{mass} + 218.63$ after acclimation ($F_{1,151}=5.368, p=0.022, R^2=0.469$). For 25//10, $\text{WBF} = -0.281\text{mass} + 252.72$ before acclimation and $\text{WBF} = -0.169\text{mass} + 224.84$ after acclimation ($F_{1,132}=7.581, p=0.007, R^2=0.542$). For 25//5, $\text{WBF} = -0.219\text{mass} + 240.17$ before acclimation and $\text{WBF} = -0.116\text{mass} + 214.68$ after acclimation ($F_{1,128}=4.484, p=0.036, R^2=0.398$).

Resting Metabolic Rate

An ANCOVA ($R^2=24.3\%$) including acclimation temperature and state, showed that body mass explained most of the variation in resting metabolic rate (RestingMR). In fact, interaction between body mass and acclimation temperature ($F_{2,392}=6.897, p=0.001, \text{partial } R^2=0.027$) (Figure 5), and the three main variables acclimation temperature ($F_{2,392}=6.800, p=0.001, \text{partial } R^2=0.027$), acclimation state ($F_{1,392}=18.360, p<0.0001, \text{partial } R^2=0.036$) and body mass ($F_{1,392}=101.093, p<0.0001, \text{partial } R^2=0.196$) were all significant. A post-hoc test showed no difference in RestingMR among acclimation temperatures, either before or after acclimation. Only bees acclimated to the control temperature (25//25) showed a

significantly lower RestingMR post-acclimation compare to pre-acclimation measurements ($p=0.004$) (Figure 6).

A 2-way ANOVA revealed that both acclimation temperature ($F_{2,14}=4.480$, $p=0.031$, partial $R^2=0.305$) and acclimation state ($F_{1,14}=6.426$, $p=0.024$, partial $R^2=0.219$) had an effect on the quantity of successful RestingMR obtained ($R^2=0.524$), i.e. the number of bees actually displaying Discontinuous Gas Exchange (see Figure 2). Post-hoc tests revealed no effect of these variables on the success of RestingMR considering each acclimation temperature at each state, but significant effects appeared when results were pooled. Indeed, with the same sampling effort, more successful RestingMR were obtained before acclimation (60.1%) than after (45.5%) ($p=0.024$). Moreover, more bees from the control group (64.3%) went into resting discontinuous gas exchange (DGE) than bees from 25//5 group (43.7%) ($p=0.033$); these results are later referred to as RestingMR success.

Maximum Nest Temperature

The effect of acclimation temperature and acclimation state on maximum nest temperature (T_{nest}) at six different time periods over 24 hours is shown in Figure 7. A 2-way ANOVA with repeated measures showed only an effect of acclimation state ($F_{1,33}=6.839$, $p=0.013$) due to a slightly higher mean T_{nest} before the experiment (40.4°C) than after a 3-week acclimation (40.1°C). The difference between T_{nest} pre and post-acclimation was not affected by acclimation temperature ($F_{2,33}=0.316$, $p=0.731$), time period ($F_{5,33}=1.101$, $p=0.378$), or their interaction ($F_{5,33}=0.511$, $p=0.870$). When analyzing T_{nest} pre and post-acclimation separately with 2-way ANOVA ($R^2=0.266$ and $R^2=0.298$ respectively), the interaction between acclimation temperature and time period showed a

significant effect on Tnest (Tnest pre-acclimation: $F_{10,361}=3.536$, $p<0.0001$; Tnest post-acclimation: $F_{10,386}=4.354$, $p<0.0001$). Post-hoc tests revealed that in most cases, Tnest slightly decreased at night in cold-acclimated groups (data not shown).

Second Cohort Acclimation Experiment

Neither acclimation temperature nor acclimation state had a significant influence on body mass of bees from the second cohort that developed under the acclimation treatments, and were measured at the ages of 1-week and 3-weeks (Table 2). An ANCOVA on the effect of acclimation temperature and acclimation state on FlightMR of the second cohort, using body mass as a covariate, showed that only body mass had an effect ($F_{1,71}=40.474$, $p<0.0001$, $R^2=0.354$). Similarly, an ANCOVA showed a significant effect of body mass, but no effect of acclimation temperature or acclimation state on WBF ($F_{1,69}=22.03$, $p<0.0001$, $R^2=0.231$). Finally body mass ($F_{1,65}=10.859$, $p=0.002$, partial $R^2=0.120$) and acclimation temperature ($F_{1,65}=4.092$, $p=0.047$, $R^2=0.045$) were found (ANCOVA) to have a significant effect on the second cohort's RestingMR ($R^2=0.284$). A significant but marginal effect of acclimation ($p=0.047$) indicated a higher RestingMR for the acclimated group 25//10 compared with the control group 25//25, probably due to bigger individuals collected in the cold-acclimated group.

Field Collection

Morphological Measurements

The body mass of workers sampled at all four latitudinal sites varied from 55.5 mg to 375.8 mg. A 1-way ANOVA revealed that body mass varied among sites ($F_{3,261}=29.017$,

$p < 0.0001$, $R^2 = 0.250$) (Figure 8A). Post-hoc tests showed no significant differences between body mass in site 1 (200.244 ± 3.229 mg) and site 4 (202.976 ± 5.948 mg), and between site 2 (142.962 ± 5.548 mg) and site 3 (151.243 ± 4.674 mg). Body mass of bees from site 1 and from site 4 were significantly different from bees from site 2 and site 3.

An ANCOVA showed an effect of both main variables (Site: $F_{3,260} = 37.802$, $p < 0.0001$, $R^2 = 0.194$; Body Mass: $F_{1,260} = 90.777$, $p < 0.0001$, $R^2 = 0.154$) on worker wing area. A post-hoc test showed differences between site 2 and site 4 ($F_{1,123} = 5.020$, $p = 0.027$) and between site 3 and site 4 ($F_{1,139} = 5.826$, $p = 0.017$).

An ANCOVA ($R^2 = 0.796$) showed that body mass explained most of the variation in wingloading ($F_{1,260} = 777.48$, $p < 0.0001$, partial $R^2 = 0.609$). The interaction between site and body mass was also significant ($F_{3,260} = 37.83$, $p < 0.0001$, partial $R^2 = 0.090$). Pairwise comparisons of the slopes revealed that the effect of body mass on wing loading is significantly different between site 2 and site 3 ($F_{1,116} = 5.430$, $p = 0.022$), between site 2 and site 4 ($F_{1,123} = 10.080$, $p = 0.002$), and between site 1 and site 4 ($F_{1,141} = 5.389$, $p = 0.022$). The relationship between wing loading and body mass of *B. impatiens* at the four sites is shown in Figure 9.

Flight Metabolic Rate

Effects of flight trial temperature and latitudinal site on field FlightMR were determined by an ANCOVA with body mass as a covariate ($R^2 = 0.570$). No linear latitudinal trend in body mass was observed, since both site 1 (northern) and site 4 (southern) workers showed highest mean body mass. This variable explained 20% of field FlightMR variation ($F_{1,254} = 118.47$, $p < 0.0001$) and its interaction with the site was also significant ($F_{3,254} = 5.96$, $p = 0.001$, partial $R^2 = 0.030$). In fact, when testing regressions at each site, all slopes of field

FlightMR on body mass were significant and between 0.032 and 0.044, at both flight trial temperatures and at all four sites, except for site 2 measurements at 25°C ($t=1.8$, $p=0.081$). Flight trial temperature explained more than 10% of field FlightMR variation ($F_{1,254}=62.73$, $p<0.0001$, partial $R^2=0.106$). Indeed, field FlightMR at 25°C (10.39 ± 0.25 mL CO₂·h⁻¹, $n=157$) was higher than at 35°C (8.36 ± 0.30 mL CO₂·h⁻¹, $n=108$) for site 1, site 3 and site 4. Site 2 measurements showed the same trends but no significant difference ($p=1.000$). The effect of flight trial temperature and latitudinal site on FlightMR of *B. impatiens* measured in the field is shown in Figure 8B.

Wingbeat Frequency

An ANCOVA was performed on the effect of site and flight trial temperature on WBF with body mass as a covariate. The final model ($R^2=0.459$) showed no significant effect of flight trial temperature on WBF (Figure 8C). Body mass explained most of the variation in WBF ($F_{1,173}=71.97$, $p<0.0001$, partial $R^2=0.225$). There was a significant effect of site on WBF ($F_{3,173}=8.32$, $p<0.0001$, partial $R^2=0.078$) but post-hoc tests showed few differences. Indeed, a Bonferroni test revealed only a significant difference in WBF between site 1 and site 4 workers flying at 35°C ($p=0.006$). A Tukey test conceded also a marginal difference between site 2 and site 4 WBF at 25°C ($p=0.040$) and between site 1 and site 3 WBF at 35°C ($p=0.054$).

DISCUSSION

No effect of acclimation

The main focus of this study was to determine whether cold-acclimated *Bombus impatiens* displays phenotypic plasticity in flight energetics. Bumblebees are heterothermic poikilotherms, meaning that they can behave as ectotherms and endotherms depending of the situation. As described earlier, many ectothermic poikilotherm insects follow the Metabolic Cold Acclimation (MCA) hypothesis, compensating for exposure to a cold environment with an increase in metabolic rate (Addo-Bediako et al. 2002). On the other hand, for endothermic homeotherms facing cold temperatures, metabolic rate (MR), mostly resting metabolic rate (RestingMR), either increases to cope with higher thermoregulatory needs, or decreases owing to thermoregulation and activity reduction. Since bumblebees are only active during a short period of the year (spring-summer season), it is unlikely that they would reduce their activity during this period. Thus, an increased MR in cold-acclimated bees was expected. Indeed, RestingMR is expected to increase in cold-acclimated animals to heat up body temperature and to improve locomotion capacities in cold environments. Since the same muscles are engaged in thermoregulation and flight, flight metabolic rates (FlightMR) were also expected to increase as these two activities are coupled. Because FlightMR is often associated with differences in wingbeat frequency (WBF), parallel changes with air temperature were expected. Nevertheless, for both acclimated cohorts, no clear effect of acclimation temperature on FlightMR and WBF were observed (Figure 3). As for RestingMR, despite significant differences among treatments detected with ANOVA, post-hoc tests showed no effect of acclimation temperature for the first cohort, and a marginally significant effect ($p=0.047$) for the second cohort (Figure 6). Therefore, it is apparent that no

metabolic plasticity occurred in cold-acclimated *B. impatiens* at least under the conditional used in these experiments. Similarly, an increase in wing size due to developmental plasticity, improving flight performance at cold temperature, is often observed in cold-acclimated *Drosophila sp.* (e.g. Crill et al. 1996, David et al. 1997, Kari and Huey 2000, Gilchrist and Huey 2004, Frazier et al. 2008), but after nine weeks of acclimation, wing loading and wing area of *B. impatiens* were not significantly different among treatments (Table 1). Finally, the “temperature-size rule” states that in most species, adult body size is bigger in individuals reared in colder environments (Kingsolver and Hedrick 2008). However, in the current study, acclimation had no effect on body mass. In summary, no metabolic or morphological adjustments occurred in cold-acclimated *B. impatiens*.

High thermoregulation of the nest

Measurements performed at the colony level shed light into the lack of acclimation of measured phenotypes. The effect of acclimation temperature on maximum nest temperature was similar in cold-acclimated colonies and control colonies (Figure 7). Even at temperatures as cold as 5°C or 10°C at night, cold-acclimated colonies displayed T_{nest} values consistently above 36.8°C (Figure 10). Therefore, brood never faced low temperatures during the experiment, and even adults on the periphery of the colony did not face temperatures as low as the imposed treatment temperatures. In essence, the colony as a whole acted like an endothermic homeotherm, where the core was maintained at constant temperature. These results agree with literature reports. It is well known that bumblebees regulate the temperature of the nest to keep the brood warm (Goulson 2010). Incubating bumblebees maintained their thoracic temperature between 34.5 and 37.5°C when the surrounding temperature fluctuated between 3 and 33°C (Jones and Oldroyd 2007), leading

to a brood temperature kept at around 30°C even in a cold environment (5°C) (Vogt 1986a). Hasselrot (1960) has also noticed that for bumblebee colonies containing many workers, nest temperature was kept constant and varied by less than 2.5°C. In the present study, T_{nest} variation ranged from 3 to 5.5°C, and these extremes were both recorded in 25//25 control colonies. Also, despite a higher number of individuals, a slight decrease of 0.3°C in average was observed in T_{nest} after three weeks of acclimation, including in the control group. Dyer and Seeley (1991) have shown that honeybee nest temperature stayed high and even more constant than bumblebee nest temperature when exposed to air temperatures as low as 12°C. Indeed, keeping the brood warm is essential in honeybees since a slight decrease in rearing temperature affects adult bee capacities, like dance performance and short-term memory (e.g. Tautz et al. 2003, Jones et al. 2005). The variation in nest temperatures observed in the present study suggests that bumblebees may not be as sensitive to brood temperature variation as honeybees, but obviously still need to keep the nest warm.

It seems that behavioural adjustments allow bees to keep the nest temperature warm at all air temperatures. Passive mechanisms, like selection of the nest site, and the orientation and architecture of the nest, help to thermoregulate the nest, but fluctuations in environmental temperature can be managed via active mechanisms (Jones and Oldroyd 2007). When temperature dropped at night, particularly at 5°C, bumblebees gathered closer to each other compared with the control colonies where ambient temperature stayed at 25°C (Figure 10). It is well known that social bees respond to low temperatures by clustering: workers stay closer or further apart depending on surrounding temperature to adjust nest temperature, since aggregating reduces heat loss by decreasing the surface area for heat exchange (reviewed in Jones and Oldroyd 2007). This behaviour is reminiscent of a pattern

observed in some endothermic homeotherms, as suggested by Southwick and Heldmaier (1987), and it might confer metabolic savings to individuals. For instance, in Abert's squirrels (*Sciurus aberti*), colder temperatures increased the number of individuals engaged in communal nesting, as predicted by the social thermoregulation hypothesis which states that endotherms will communally nest to reduce thermoregulatory costs in cold environments (Edelman and Koprowski 2007). Muskrats that display overwintering social aggregation follow a similar trend, with a lower RestingMR than single counterparts (Bazin and MacArthur 1992).

In contrast to a honeybee cluster that contains tens of thousands of individuals that can heat up the brood, and create a barrier all over the nest, a bumblebee colony hardly reaches 400 individuals. Southwick and Heldmaier (1987) suggested that more bees in the nest results in a smaller proportion of the colony that has to be exposed to the environment. It has been proposed that bumblebees use futile cycles between fructose-6-phosphate and fructose-1,6-biphosphate to produce heat without muscle movement (Newsholme et al. 1972, Clark et al. 1973), whereas honeybees would rarely use this mechanism because only small quantities of the necessary enzymes, particularly fructose diphosphatase, were found in this species (Newsholme et al. 1972). This difference could help small bumblebee colonies to survive cold temperatures, and allow bumblebees to fly under cold weather, in contrast to honeybees (Newsholme et al. 1972). However, the existence of this mechanism in bumblebees has been recently questioned (Staples et al. 2004). Contrary to what has been observed in the present study, enhanced thermoregulatory work in cold-acclimated colonies, either by futile cycles or mechanical heat production, would have suggested higher RestingMR or flight metabolism adjustments. The results of the present study suggest that

metabolic phenotype of adult bumblebees is not plastic, and slight variations in nest temperature do not seem to disturb metabolic development in egg, larvae and pupae of bumblebees.

Cold-acclimated colonies almost always had a T_{nest} as high as control colonies (Figure 7), leading to the absence of differences in metabolic or morphometric variables of individual bumblebees among the treatments. But, keeping the nest temperature as high at 5°C as at 25°C may have a cost. Three observations made during the present study corroborated the hypothesis of an increased thermoregulatory cost for colonies facing cold temperatures.

First, RestingMR success (i.e. the percentage of bumblebees that effectively rested and showed discontinuous gas exchange (DGE) during RestingMR measurements) was higher for individuals from the control incubator (64.3%) than for 5°C-acclimated bees (43.7%). This may illustrate that cold-acclimated individuals were more stressed and that it was more difficult for them to achieve a resting state. The nature of this observation remains unclear and has to be investigated.

Second, in all three 25//5 colonies, males and future queens hatched earlier (hence, these colonies could not be used in the second cohort experiment). These observations may have three possible explanations. *i)* Some colonies may have been shipped older than others, since the company from which they were purchased has a selling criterion based on the number of workers in the colony and not on the age of the colony. It is however unlikely that all three colonies designated as 25//5 were older than the six other ordered colonies. *ii)* The fact that it was cold at night may have triggered mechanisms associated with winter arrival or summer shortness, and prompted the end to the breeding season with the production of

sexuals. Photoperiod, food availability and precipitation are known to have an effect on the development of a bumblebee colony (de la Hoz 2006, Amin et al. 2007), and these variables were kept constant through all treatments in the present study. However, it is unclear whether temperature has a direct effect on the colony cycle (Vogt 1986b). *iii*) The last explanation would be that cold-acclimated colonies aged faster and particularly that the queen was exhausted earlier. Even though previous research showed that thermal conditions had no effect on bumblebee worker mortality (Vogt 1986b), brood incubation is expensive, particularly at near freezing ambient temperature. At 5°C, mass-specific MR of incubating bees is as high as that of flying bees, whereas when air temperature is near 35°C, incubating MR is hardly above RestingMR (Heinrich 1993). Besides being energetically expensive, maintaining a warm nest has a high cost in time since it assigns many workers to the incubation task. Colony thermoregulation at 5°C recruits almost all workers in the nest, leaving only 10% of them to take care of all the other tasks in the colony, such as brood monitoring, cleaning, larvae feeding and honey pot filling (Vogt 1986a). This reduction in brood maintenance may explain lower growth rates in colonies acclimated to 15°C versus 25°C (Vogt 1986b), especially as more bees induce a temperature increase solely through their presence (Vogt 1986b). Heinrich (1974) calculated that it would not be possible for bees to incubate as much in nature, where food supply is limited, as they do in the laboratory. However, he noted also that bumblebee nests in the field are well insulated, considerably reducing metabolic needs.

Third, a noteworthy observation made in the present study was that two out of three colonies acclimated to 5°C during the night built wax walls around the brood (personal observations). Since colonies were disturbed regularly to collect bees for the experimental measurements or to feed the bees (pollen balls were changed at least every week), they may

have built these fences as a physical barrier to protect the brood. However, despite being disturbed to the same extent, none of the six 25//10 and 25//25 colonies displayed this behaviour, leading to the hypothesis that the 25//5 colonies built these walls as a thermal fence to reduce heat loss from the nest. This phenomenon has been observed previously stated and called a “wax canopy”, but it has not been studied. A photograph of this construction is presented in Schultze-Motel (1991), where a short sentence explains that bumblebees covered thermocouples that previously seemed to annoy them and ended up using them as building foundation. In this case, it was either a way to discard a foreign and unwanted object, or to use any available item to add strength to the nest-building. The same phenomenon was described by Heinrich (1979) as a thermal protection to increase nest insulation. He observed bumblebees using any piece of material they could find to insulate the nest. He added that they often covered the nest with a wax roof to trap heat coming from the bees and limit heat loss. He also noticed that this behaviour seemed plastic, since the bees would also pull the roof down when there was a risk of overheating. Insulation is crucial to colony health. Uninsulated colonies kept at 15°C have exhibited lower nest temperatures and have produced fewer bees than insulated colonies at 15°C (Vogt 1986b). Colony cycle variables of insulated colonies reared at 15°C were identical to those of uninsulated colonies reared at 25°C (Vogt 1986b). As discussed earlier, insulation of the nest is essential to allow time for foraging and brood maintenance.

It is interesting to point out that in these three observations (RestingMR success, early production of sexuals, and thermal fence building) the differences apply between 25//5 and 25//25 colonies, but not between 25//10 and 25//25 colonies. A threshold between 5 and 10°C may exist, at which point it is clearly more demanding for the colony to keep the brood warm. The chill-coma temperature in bumblebees is around 7°C (Goller and Esch 1990),

which is probably the threshold below which it is unsustainable for uninsulated colonies to survive in nature.

Absence of latitudinal trend in measured variables

As suggested by recent literature, physiological responses studied under controlled laboratory conditions do not necessarily represent the adjustments happening in natural environments (Overgaard et al. 2010). Hence, the second main goal of the present study was to evaluate and compare flight energetic physiological and morphological parameters among populations of *B. impatiens* distributed along a latitudinal gradient, and exposed to diverse climates. A review of studies comparing physiological features between populations of vertebrates (both ectotherms and endotherms) concluded that there is often variation between populations of animals, usually suggesting genetic variation (Garland and Adolph 1991). In invertebrates, several papers showed physiological adjustments between populations, usually with smaller body size, and either higher MR (Lardies and Bozinovic 2006, Whiteley et al. 2011) or lower MR (Berrigan and Partridge 1997, Lachenicht et al. 2010) in low-latitude populations. Therefore, a latitudinal pattern for FlightMR, body mass and wing measurements in *B. impatiens* was expected. However, no latitudinal trend was observed for any of the measured variables.

B. impatiens collected at four latitudes (45°, 41°, 37°, 34°) showed similar mass-specific FlightMR. These results contrast with the higher mass-specific FlightMR detected in African honeybees (*Apis mellifera scutellata*) compared with European subspecies (Harrison and Hall 1993, reviewed in Harrison and Fewell 2002), suggesting a possible link with conditions associated with latitude. However, the effect of latitude alone is uncertain in this case as African and European honeybees are classified as subspecies. The lack of latitudinal

difference observed in *B. impatiens* in this study can be explained by the previous conclusion that adult bumblebees do not exhibit thermal plasticity, and that they maintain similar brood temperature in all environments, helped by more or less insulation. Initially, morphological measurements were also predicted to vary along the latitudinal gradient, but they did not, even if this trait seems plastic in other bee species. In honeybees (*Apis mellifera*) in Africa, populations from high altitudes exhibit lower wing loading due to bigger wing area for a similar body size (Hepburn et al. 1998). However, honeybees from northern Europe have higher wing loading than South Europe bees, because they have to lift heavier bodies with the same wing surface area (Hepburn et al. 1999). A review of studies on Bergmann's rule showed that a small majority of research, including studies conducted on bumblebees, supports the pattern describing the presence of larger individuals in colder climates at both the species and the population levels (Watt et al. 2010). In the present study, bumblebees do not follow Bergmann's rule since individuals from intermediate latitudes were smaller than counterparts from both ends of the transect. Also, no latitudinal trend emerged from wing measurements. The relationship between body mass and wing loading had a steeper slope at site 2 than at site 3 and site 4. This observation seemed opposed to that detected in other insects where wing loading decreases at higher latitudes or altitudes (Azevedo et al. 1998, Hepburn et al. 1998). These results came from one sampling time from a particular year, at one location at each latitude, and differences may arise from local and/or temporal conditions, such as climate and resources quantity and quality, or from genetic innovation arising in a particular region. A study on the common terrestrial isopod, or common woodlouse (*Porcellio laevis*), showed no latitudinal trend of the adult body mass across four latitudes (Castañeda et al. 2005), whereas the same research group observed a significant relationship between the instar size and five latitudes in another study (Lardies and

Bozinovic 2006). The difference may be linked to the development stage, or, as the authors mentioned, may be the consequence of other factors such as food availability or an insufficient number of compared populations in the first study (Castañeda et al. 2005).

Major effect of body mass

Contrary to acclimation and site factors, effects of body mass followed the expected pattern. Body mass explained most of the variation for all measured variables, morphometric, physiological or wing kinematic, both for young individuals and for the older cohort, and from both laboratory and field measurements (Table 1). Bigger individuals had higher RestingMR, FlightMR, wing area and wing loading, and lower WBF. A strong positive correlation between body mass and MR has long been observed in flying insects at rest and in flight as an allometric relationship at the interspecies level (Bartholomew and Casey 1978, Darveau et al. 2005), and as an isometric relationship within species (Skandalis and Darveau, submitted). Studies showing no effect of total body mass, for example on honeybee FlightMR (Feuerbacher et al. 2003), are rare and they may be explained by a narrow body size range. Wingbeat frequency is also usually inversely correlated to body mass as shown in the present study, but seems to be mostly explained by wing measurements such as wing length, wing area or wing loading (Bartholomew and Casey 1978, Joos et al. 1991, Darveau et al. 2005, Skandalis and Darveau, submitted), which was not studied here.

Strong effect of air temperature on flight metabolic rate

In flying insects, temperature effects on FlightMR depend on the thermal strategy of the species (reviewed in Harrison and Roberts 2000). In ectothermic small insects such as *Drosophila*, oxygen consumption increases with temperature (e.g. Yurkiewicz and Smyth

1966). In endothermic species, MR decreases while air temperature increases (e.g. Roberts and Harrison 1999). Since bumblebees are endothermic poikilotherms, FlightMR was expected to decrease with temperature, and this pattern was in fact observed. Both during the acclimation experiment and in all four field sites, FlightMR decreased when ambient air increased (Figure 3), and the percentage variation in FlightMR explained by the variation in flight trial temperature was high. FlightMR at 15°C was two-fold greater than it was for bumblebees flying at 35°C (Figure 3A and Figure 8B). Similar observations have been made in the bee *Centris pallida* between 26 and 35°C (Roberts et al. 1998), and in honeybees between 20 and 40°C (Harrison et al. 1996). The latter study showed that FlightMR in hovering undisturbed honeybees decreased by 40% when the air temperature increased from 20 to 40°C. Close to what was observed in the present study, this pattern can be explained by the fact that all these species are endothermic poikilotherms and increase thoracic temperatures to near 35°C before takeoff (see Heinrich 1975 for bumblebees). This involves harder work to heat up their thoracic flight muscles at colder temperatures, leading to higher FlightMR. However, the trend was not observed in all studies performed on Apidae. Heinrich (1975) showed no correlation between MR of two bumblebee species (*B. vosnesenskii* and *B. edwardsii*) and the ambient temperature between 10 and 32°C, and in honeybees between 20 and 42°C (Heinrich 1980). Early on, Allen (1959) demonstrated a change in the effect of surrounding temperature on MR depending on the age of honeybees. Overall, she observed a positive correlation at temperatures colder than 17°C, followed by a small decrease of MR with the temperature of the air, then a plateau for bees flying at 27 to 32°C, and ending by a positive correlation at warm air temperatures.

As Woods et al. (2005) pointed out, studies on honeybees showed positive, negative and non-existent relationships between FlightMR and surrounding temperature. They first suggested, based on previous studies, that these inconsistent results may be the consequences of different metabolic capacities linked to variation between colonies, genotypes, seasons, and foraging task (Woods et al. 2005). The authors further suggested that MR would depend on bee willingness to fly. In fact, they first observed an inverse relationship between FlightMR and air temperature when they pooled all their data. But, when looking only at “first-quality flight” (i.e. honeybees that continuously flew, without shaking the chamber), FlightMR was independent of ambient temperature (Woods et al. 2005). The same conclusion was derived from unshaken honeybees, compared to shaken bees. Again, the longer the bees spent in flight, the lower the effect of surrounding temperature on MR. Analyzing both thoracic and air temperatures, they concluded that FlightMR depended on the thoracic temperature, instead of the ambient temperature (Woods et al. 2005). Their interpretation was that at a thoracic temperature below 38°C, a positive relationship is observed, while when the thorax is maintained above 38°C, a negative relationship occurs. Using only Type 1 flight data from the present study, which used similar criteria to the “first-quality flight” reported by Woods et al. (2005), FlightMR was still strongly linked to flight temperature (results not shown), suggesting that flight thermoregulation in bumblebees might be different than in honeybees.

Furthermore, using honeybees Woods et al. (2005) also reported greater successful flight when flying at colder temperatures, while bumblebees in the present study showed lower success rates when flying at colder temperature (15°C) (personal observations). Heinrich (1993) showed that it took close to five minutes for the thorax to reach a temperature above 35°C for a *Bombus vosnesenskii* queen placed at 13°C. Heinrich (1975)

also found that continuous flight was not maintained by honeybee workers when the ambient temperature dropped below 10°C, because their thoracic temperature declined to 30°C or less. While, at all flight trial temperatures in the present study, it seemed that smaller bumblebees were not as successful to fly as bigger bees, it was not obvious they were flying less at cold temperature than their bigger counterparts, contrary to previous predictions (Heinrich and Heinrich 1983).

In addition, *B. impatiens* collected and measured outdoors in the field, at all four latitudes, flew more consistently in the respiratory chamber than individuals from a commercial supplier measured indoors (personal observations), which supports literature reporting that honeybees were more willing to fly when they were measured outdoors than indoors (Woods et al. 2005).

Finally, some species maintained in laboratories for generations and/or raised in laboratories tend to be differently affected by surrounding temperature than in the field (Steigen 1976, Young 1979), often exhibiting a reduced MR after a prolonged time in the laboratory (e.g. Young and Block 1980, Terblanche et al. 2004). This reduction may be related to a more constant thermal habitat and decreased activity due to the absence of predators and constant access to food (Chown and Terblanche 2006). Trends in the present study did not follow the literature. At 25°C, mass-specific FlightMR tended to be higher in the laboratory than in the field; at 35°C, values were similar to slightly lower when recorded in the laboratory compared with the field (Figures 3 and 8). Therefore, no clear difference appeared between field populations and commercial colonies in this study.

No effect of air temperature on wingbeat frequency

In flying insects such as Diptera, it has long been accepted that wingbeat frequency increases with ambient temperature (Unwin and Corbet 1984) and is a good indicator of energy consumption (Sotavalta 1952). On the other hand, in Hymenoptera, the effect of air temperature on wingbeat frequency (WBF) remains unclear. Most articles report either an inverse correlation or an absence of relationship between the two variables; others, such as Woods et al. (2005) have shown an increase in honeybee WBF with air temperature, particularly between 19 and 25°C, and have proposed that the pattern was due to a thoracic temperature below 36°C. This explanation is at odds with previous results obtained on bumblebees showing no effect of variation in thoracic temperature on WBF (Joos et al. 1991). Most studies on bees have reported decreasing WBF with rising air temperature (Unwin and Corbet 1984, Spangler and Buchmann 1991, Harrison et al. 1996, Roberts et al. 1998, Borell and Medeiros 2004) and have proposed that this mechanism regulates heat production in order to reach thermal stability in thoracic muscles. In the present study, both during the acclimation experiment and in the field, WBF was unaffected by trial temperature (Figure 3B and Figure 8C). This temperature independence of WBF has been previously mentioned (Sotavalta 1952, Joos et al. 1991, Harrison and Roberts 2000). Several mechanisms may explain a decreased FlightMR despite a constant WBF when air temperature increases. For example, passive action of higher surrounding temperatures on the flight muscle system may ease the wing movement, notably by increasing velocity of cross-bridges formation, i.e. the temporary links between myosin and actin filaments during flight muscle contraction. A warmer thorax may also increase elastic energy storage in the muscle or the cuticle, diminishing the power required to beat the wings and improving the overall flight efficiency (Roberts et al. 1998, Harrison and Roberts 2000). In addition, Sotavalta (1952)

proposed that it would be energetically cheaper to vary the amplitude rather than the frequency of the wingstroke. Experiments on bees flying in gases of various densities showed that bees usually maintain WBF constant and adjust stroke amplitude. In heliox, a 21%:79% oxygen/helium mixture with three times lower density than ambient air, orchid bees (*Eulaema meriana*, *Euglossa imperialis*, *Euglossa dissimula*) and honeybees increased wing stroke amplitude by 30 to 50%, but kept the same WBF (Dudley 1995, Altshuler et al. 2005). Another study on carpenter bees (*Xylocopa varipuncta*) performing flight in gases of various density, such as heliox, showed that the amplitude had more capacity to vary (10 to 30%) to maintain the body aloft in the low-density air than wingbeat frequency (0 to 8%) (Roberts et al. 2004). Bumblebees measured in the present study might vary wingbeat amplitude instead of frequency to compensate for changes in ambient air temperature.

Aging effect on bumblebee physiology

Senescence of insect flight capacities has been studied for more than half a century. In the present study, the control group displayed no variation between 1-week old and 3-week old bumblebees during the second cohort experiment (Table 2), but showed lower RestingMR and FlightMR at the end of the adult acclimation experiment of the first cohort (Figures 3A and 6). These differences could be associated with a decline in metabolic properties with age, where individuals after three weeks of acclimation were at least three weeks old, and likely older than four weeks old for most. Most studies have observed a quick increase in flight performance in the first couple of days after adult emergence, and a decrease in old individuals, notably in the duration of sustained flight (Baker 1976). This decrease has been linked to a reduction in neurological, physiological and biochemical parameters (Baker 1976), such as lower rate of incorporation of leucine into proteins

(Crailsheim 1986), lower glycogen synthesis (Neukirch 1982), and 35% decrease in brain cell quantity (Rockstein 1953) found in old honeybees, and cytosolic changes (Sohal 1976) and mitochondrial damages observed in old flies (Miller et al. 2008). Similarities between species, for example in reduced glycogen concentration in both old Hymenoptera and Diptera (Williams et al. 1943, Neukirch 1982), suggests the aging process of flight capacities may be shared between most species and follows a common ordered program (Baker 1976). The effect of aging appears through various observations. For example, worker honeybees have more difficulties in learning new foraging information at the end of their life (Tofilski 2000), and maximal flight capacity in low density gases starts to decrease at 30-days of age (Vance et al. 2009). Some studies suggest that changes in older honeybees may be more dependent on behaviour, like foraging experience, than on age (Withers et al. 1993, Roberts and Elekonich 2005). The same senescent pattern is expected from FlightMR measurements but no study has specifically looked at this parameter late in the life of bees. Since reduced neurological, physiological and biochemical systems would likely slow metabolism on top of diminishing locomotor capacities, RestingMR is also expected to decrease with age, as it does for the male house cricket (*Acheta domesticus*) (Hack 1997) and the potato beetle (*Leptinotarsa decemlineata*) (Piiroinen et al. 2010). Allen (1959) observed a sharp increase in RestingMR in young honeybees, followed by a slight increase from 14-day old to 33-day old individuals at all air temperatures. A similar pattern described an increase in FlightMR in honeybees during the first days of foraging, followed by a plateau in the following ten days (Schippers et al. 2010). Again, mass-specific heat production appeared to increase greatly with age in 0 to 20-day old honeybees and seemed to remain constant afterwards (Fahrenholz et al. 1989). However, the decline in metabolic rate, at rest and during flight, has not been studied during senescence so the difference observed in the present study

cannot be directly compared with literature values. Nevertheless, decline in various aspects of physiology related to flight suggests that such a reduction would be observed.

As a result, wingbeat frequency is expected to follow the same trend of increasing in young individuals and falling in old flying insects. Effectively, 100% of WBF capacities is achieved during the first three days of adult life in *B. impatiens* (Skandalis et al. 2011). Later in the life of flying insects, Rockstein et al. (1966) observed a decrease in flight duration and a slightly reduced WBF in old houseflies (*Musca domestica*). In *Drosophila melanogaster*, WBF significantly decreased between the 42nd and the 49th day of their lives, and they could not fly at all on day 56 (Miller et al. 2008). In the present study, no difference between 1-week and 3-week old bee WBF was detected in the second cohort, but the interaction between the state and body mass was significant in the first cohort. Indeed, the negative correlation was stronger before than after the acclimation experiment for all groups, including the control group. Therefore bigger bees tend to have more similar WBF to small bees when they were older, despite a potentially higher wing loading in older bees due to damaged wings. Bigger honeybees are in fact known for lower WBF but larger stroke amplitude than small ones (Vance et al. 2009). When exposed to low density gases to reach maximal flight capacities, older honeybees also tended to decrease WBF and increase the wing amplitude (Vance et al. 2009). In summary, even if more research is necessary, bumblebees seem to keep a constant wingbeat frequency (Skandalis et al. 2011), and possibly resting and flight metabolic rates throughout most of their lives; however, during their last days, senescence of various aspects of their physiology may lead to decreased metabolism and lower flight ability.

Conclusions and future directions

Contrary to most endothermic homeotherms and ectothermic poikilotherms, the heterothermic poikilothermic bumblebee *B. impatiens* displayed no phenotypic plasticity of metabolic traits when facing cold acclimation, as shown in the present study. Although bumblebees are temperature generalists that can fly on freezing days as much as in subtropical climates, by adjusting metabolic rate to compensate for surrounding air temperature variation, the question remains whether individuals facing cold temperature can acclimate. The whole bumblebee colony acts as a temperature specialist organism, maintaining a nearly constant brood temperature despite environmental fluctuations. The increased thermoregulatory demands associated with temperature regulation did not impact individual metabolic phenotypes, but several behavioural changes appear to help them cope with temperature variation. Individuals developing in variable thermal habitats did not exhibit phenotypic changes as they appeared to be maintained in stable thermal conditions.

Nevertheless, it would be interesting to know whether individual thermoregulatory capacities have been altered by cold-acclimation. First, can cold-acclimated bumblebees reduce heat loss and start flying faster at cold air temperature? Cooling-down rate after flight and warm-up rates after cold exposure have been measured on all bees in the present study and these data may help to address such questions. Moreover, if bumblebees could not warm-up their body faster, it would also be relevant to measure the thoracic temperature at which cold-acclimated bees can fly. They may be able to take off earlier than other bees, due to lower thoracic temperature requirements. Surprisingly, acclimation to warm temperature often increases body temperature in endotherms such as mammals and birds (reviewed in Boyles et al. 2011). Finally, a research on hair length and density and associated thermal

conductance of cold-acclimated and/or high latitude bumblebees would help evaluate the energetic contribution of this trait to bumblebee thermoregulatory capacities. In *Colias* butterflies, the fur is an efficient insulation parameter to reduce convective heat loss (Kingsolver and Moffat 1982) and individuals from higher altitudes have thicker pile (Kingsolver 1983). The energetic importance of fur has also been observed in honeybees (Southwick and Heldmaier 1987). Bee species found in the tropics have few short hairs or no pile at all, whereas bumblebees are known for their hairiness, which led Heinrich (1993) to wonder if this furriness is an adaptation to cold weather, or if southern glabrous bees are adapted to warm climates. Head and thorax fur provides required insulation to bumblebees to allow them to fly at low temperature (Church 1960). Within a species, hair density appears to have more insulation effect than hair length (Church 1960). In a study on Eurasian honeybees, the northern population exhibited longer hair on tergite 5 than the five more southern populations studied (Adl et al. 2007). Further studies on North American bumblebees are required to better understand the thermal biology of these species that are essential to the ecosystem pollination.

Owing to the human-induced climate change now being faced, improving scientific knowledge of evolutionary physiology is critical (Chown et al. 2010). Since the 1960s, most habitats on Earth have warmed or cooled by 0.3 to 1°C every decade (Walther et al. 2002). Temperature affects most biological parameters, from chemical reactions to interactions between individuals of the same species, or among species. Hence, global climate change has already started to alter community structure and dynamics and will continue to do so. To successfully manage biodiversity in the future, we need to predict the consequences of climate change and other anthropological alterations of ecosystems on plant and animal

populations (Chown and Terblanche 2006). That is, we need to understand the effects of abiotic factors (e.g. temperature) and the effects of their spatial and temporal fluctuations on individuals and on organisms' interactions with their surroundings. Hopefully, closer collaboration between evolutionary physiologists and conservation biologists in the future will help quick and relevant actions to be taken to face this growing common challenge. The results of the current study aimed to take part into this wide and urgent project requiring increased thermal biology knowledge (Angilletta et al. 2006, Chown et al. 2010, Boyles et al. 2011). It appears as though *B. impatiens* may not be directly affected by a variation in environmental temperature since the colony can adjust to extreme temperatures by modifying nest insulation. However, bumblebee interactions with other species, like pollinated plants or parasites, may be widely impacted if distribution, abundance or phenology are quickly shifted, leading to a decrease or even an extinction of bumblebee species. In turn, bumblebee population alteration would have dire consequences on ecosystems and on human economy (Losey and Vaughan 2006). Climate change is not the only nature deterioration with anthropogenic roots that will have dramatic consequences on community equilibrium. For example, habitat destruction, pesticide use, soil and water pollution, invasive species introduction, may also induce major damage to bumblebee populations (Chown and Terblanche 2006) and their consequences have to be rapidly studied in order to minimize future repercussions and develop appropriate approaches.

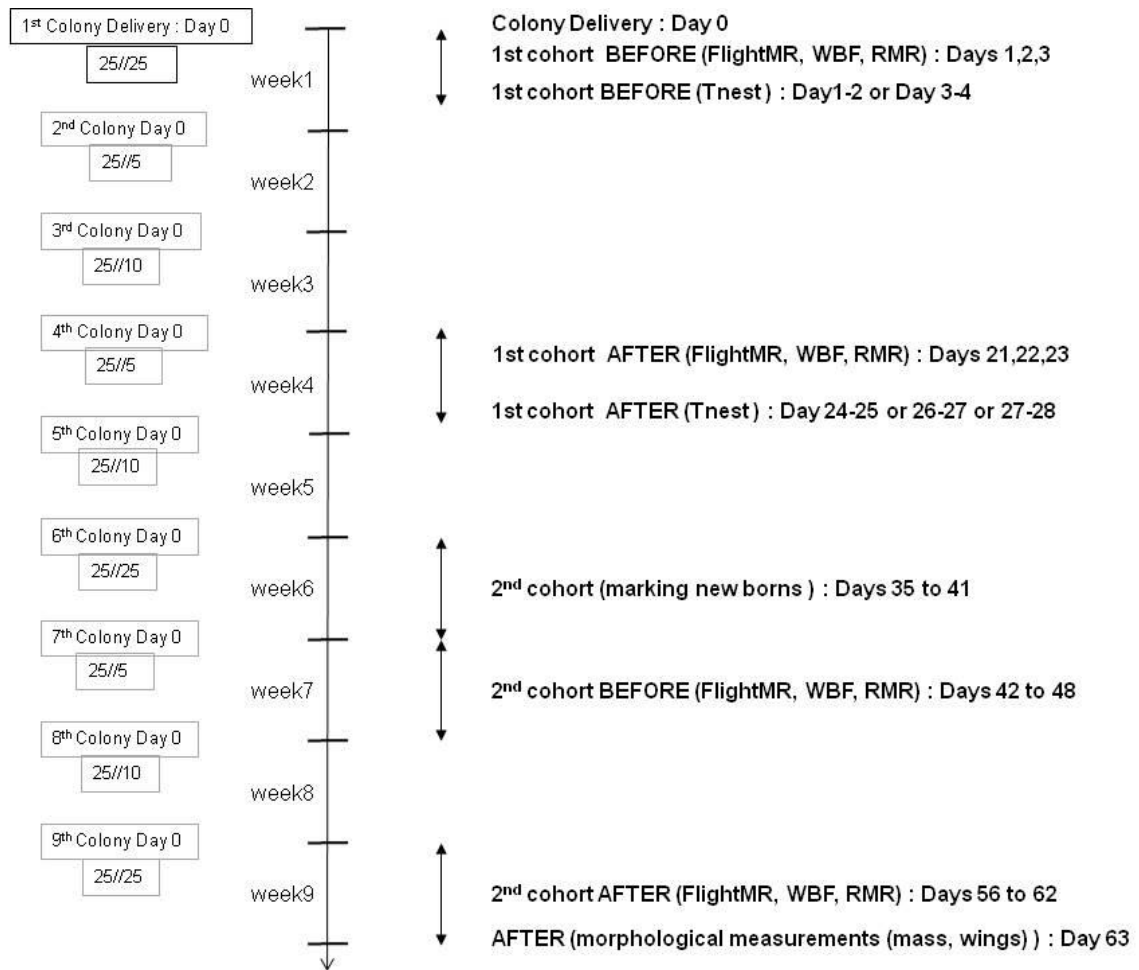


Figure 1. Acclimation experiment timeline followed for each of the nine colonies. One colony per week was received, and each colony was acclimated for nine weeks. Adult acclimation was studied through measurements on the first cohort, pre-acclimation (before) and post-acclimation (after). Pre and post-acclimation measurements on the second cohort focused on the study of the effect of acclimation during both development stages and adult stage.

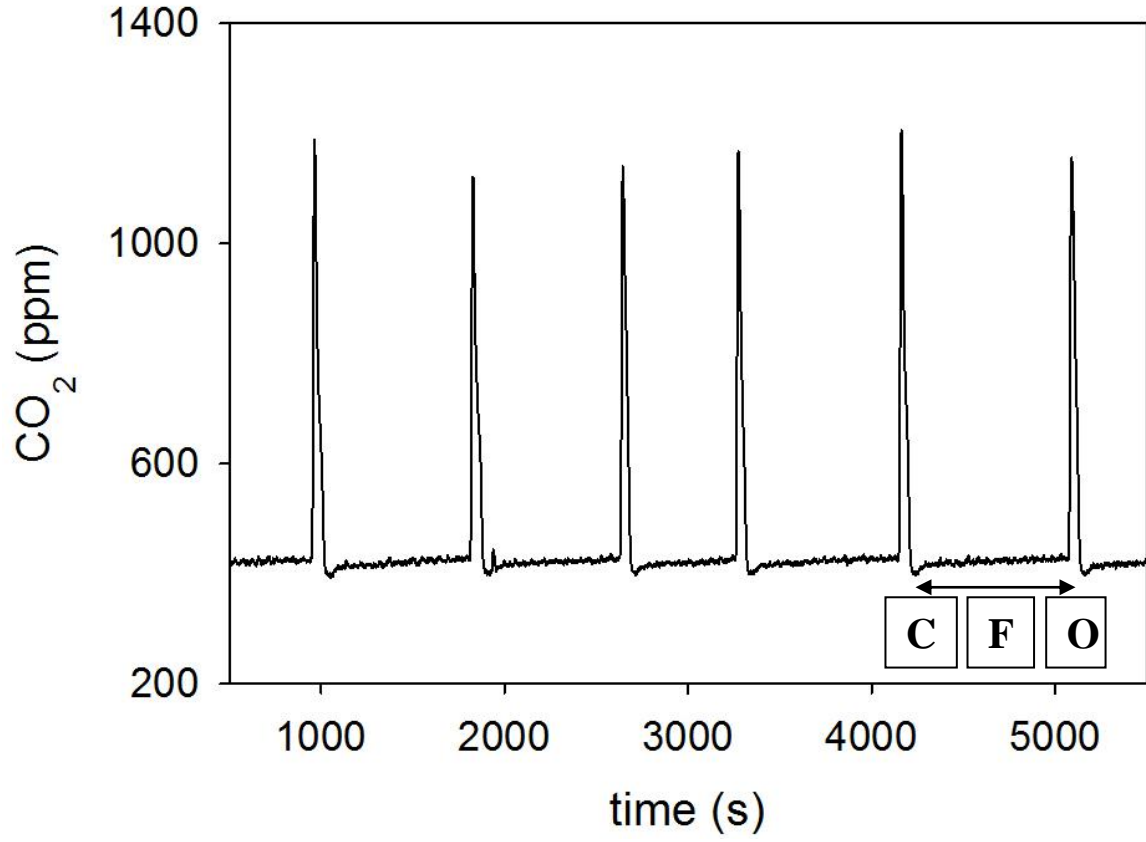


Figure 2. Representative trace of discontinuous gas exchange (DGE) in *Bombus impatiens* used as an indicator of resting state for resting metabolic rate measurements. A full cycle of DGE consists of three phases corresponding to the spiracles' state (Chown et al. 2006). The C-phase where spiracles are closed leads to the absence of respiratory exchange with the environment. During the F-phase, or fluttering-phase, spiracles are irregularly opening and closing. Lastly, the burst O-phase allow spiracles to fully open, releasing a large amount of CO₂ in a burst.

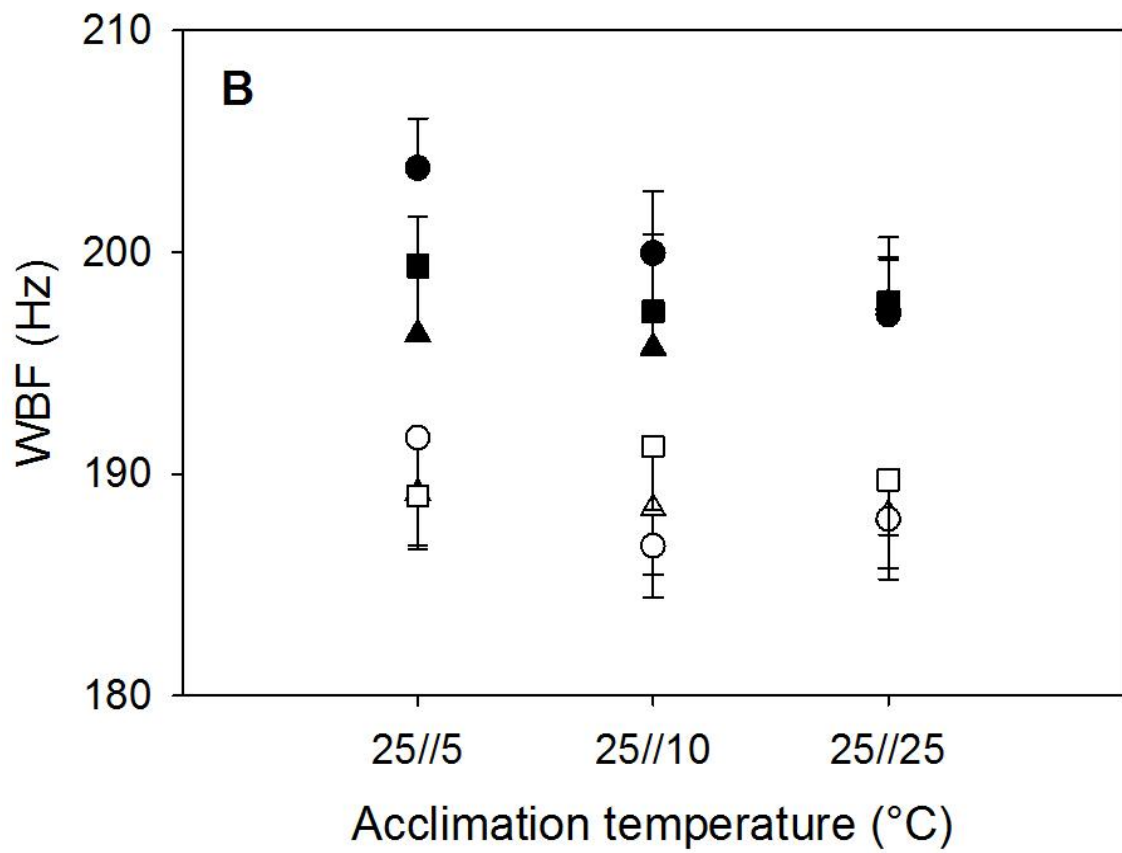
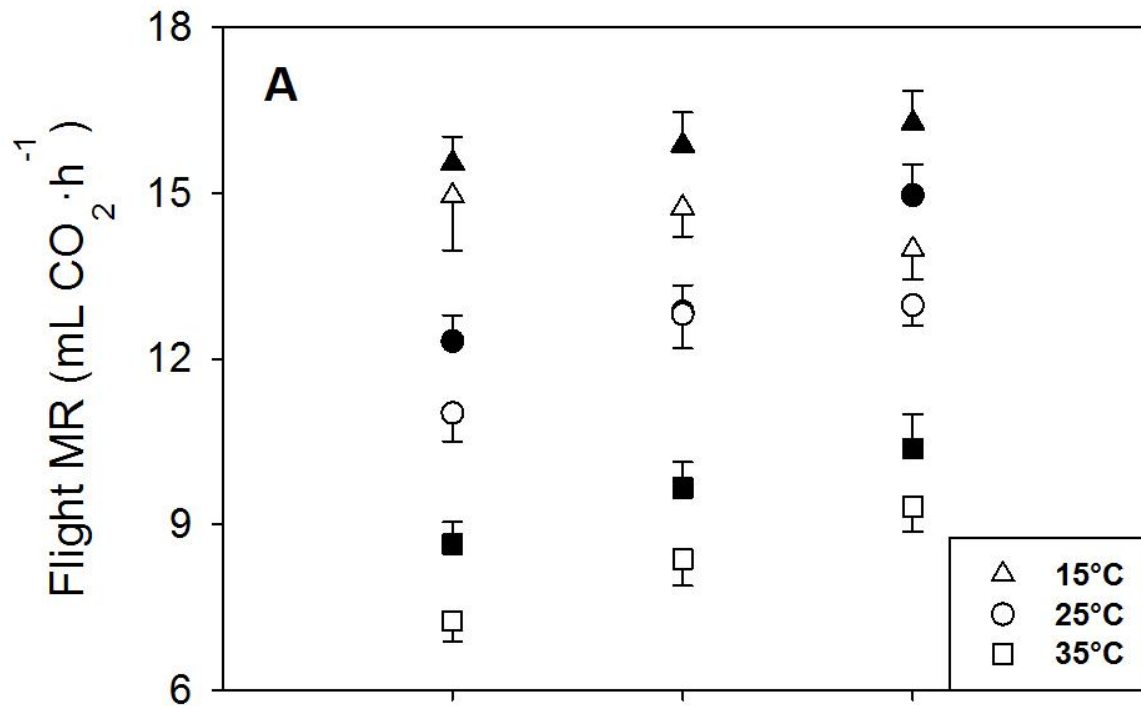


Figure 3. Mean \pm SE flight metabolic rate (FlightMR) ($\text{mL CO}_2 \cdot \text{h}^{-1}$) (panel A) and wingbeat frequency (WBF) (Hz) (panel B) at three flight trial temperatures (different shaped symbols), before (filled symbols ●) and after (open symbols ○) acclimation at three different acclimation temperatures. Sample size varies from 20 to 43 worker bees for each group on the panel A, and from 17 to 32 workers for each group on the panel B. A significant effect of flight trial temperature ($p < 0.0001$) and acclimation state ($p < 0.0001$) on FlightMR was detected in almost all cases, whereas acclimation temperature had almost no significant effect. Flight trial temperature, acclimation state and acclimation temperature had no significant effect on WBF. See Results for details.

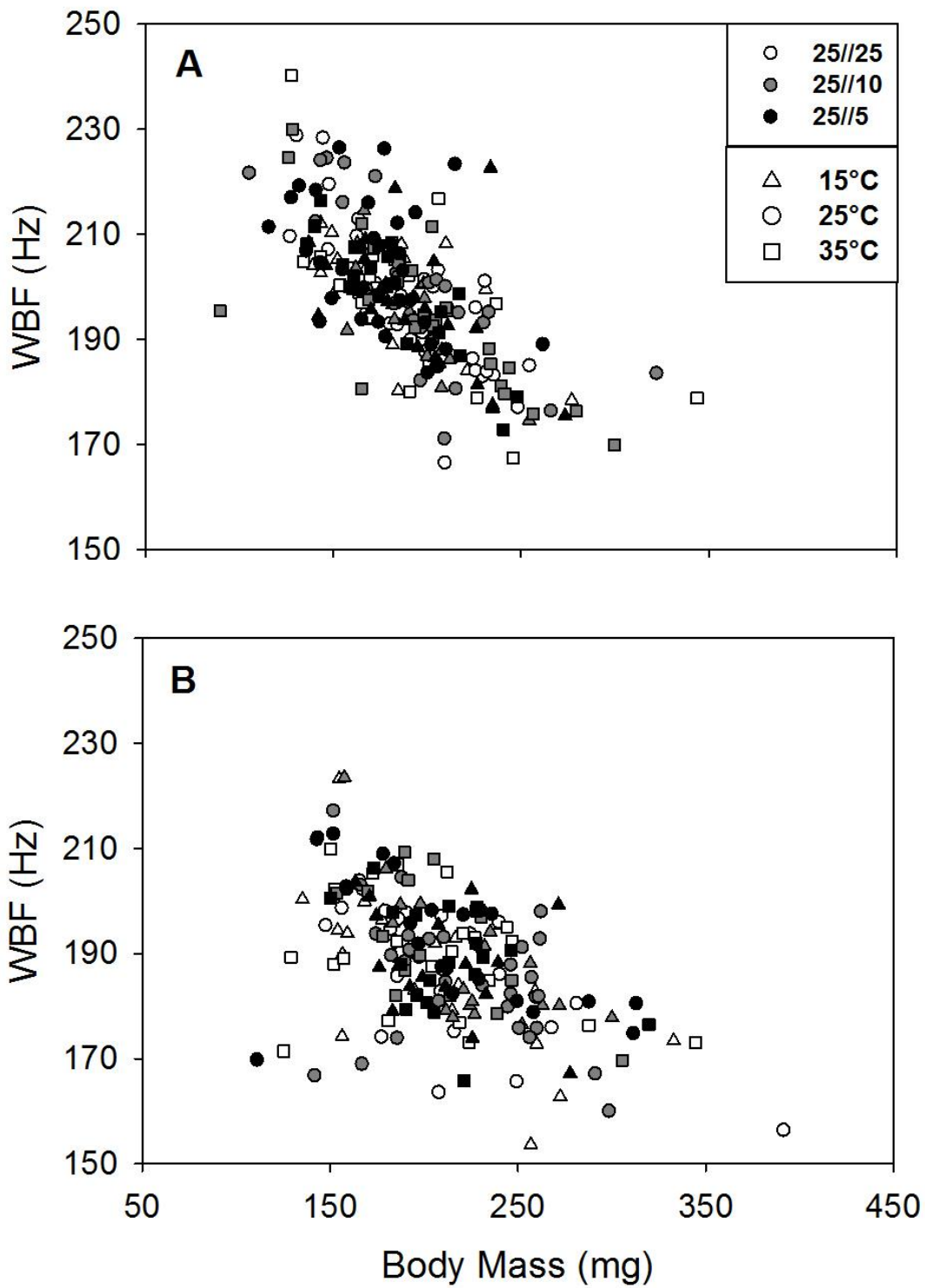


Figure 4. Relationship between wingbeat frequency (WBF) (Hz) and body mass (mg) of *B. impatiens* individuals measured at three flight trial temperatures (different shaped symbols), before (panel A) and after (panel B) acclimation at three different acclimation temperatures (different shade symbols). An ANCOVA describing the effect of the acclimation state and body mass on WBF of bumblebees showed a different slope between pre and post acclimation states for all three acclimation temperatures.

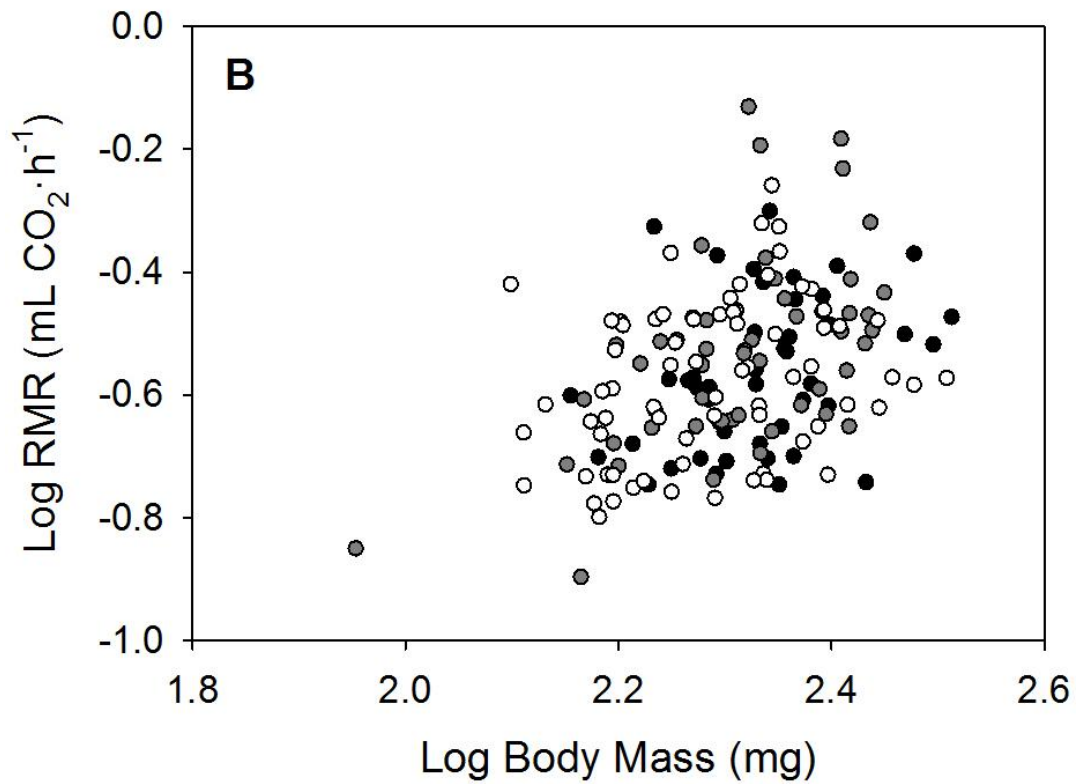
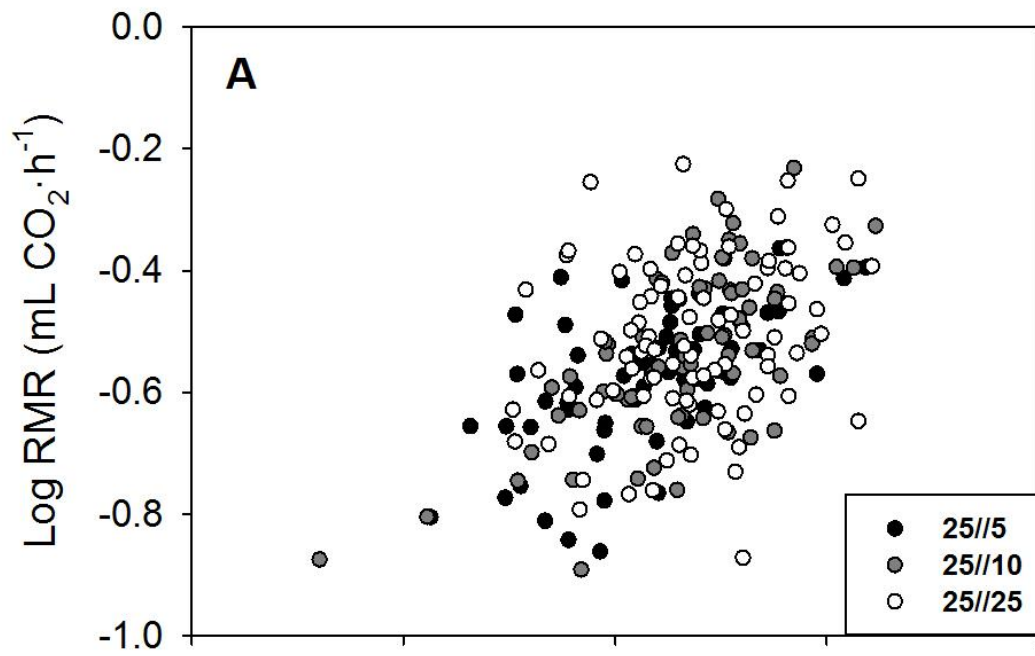


Figure 5. Relationship between log-transformed resting metabolic rate (RMR) (mL CO₂·h⁻¹) and log-transformed body mass (mg) of *B. impatiens* individuals before (panel A) and after acclimation (panel B) at three different acclimation temperatures (different shade symbols). An interaction between body mass and acclimation temperature ($F_{2,392}=6.897$, $p=0.001$, partial $R^2=0.027$) was significant, but no clear difference can be observed from the clusters.

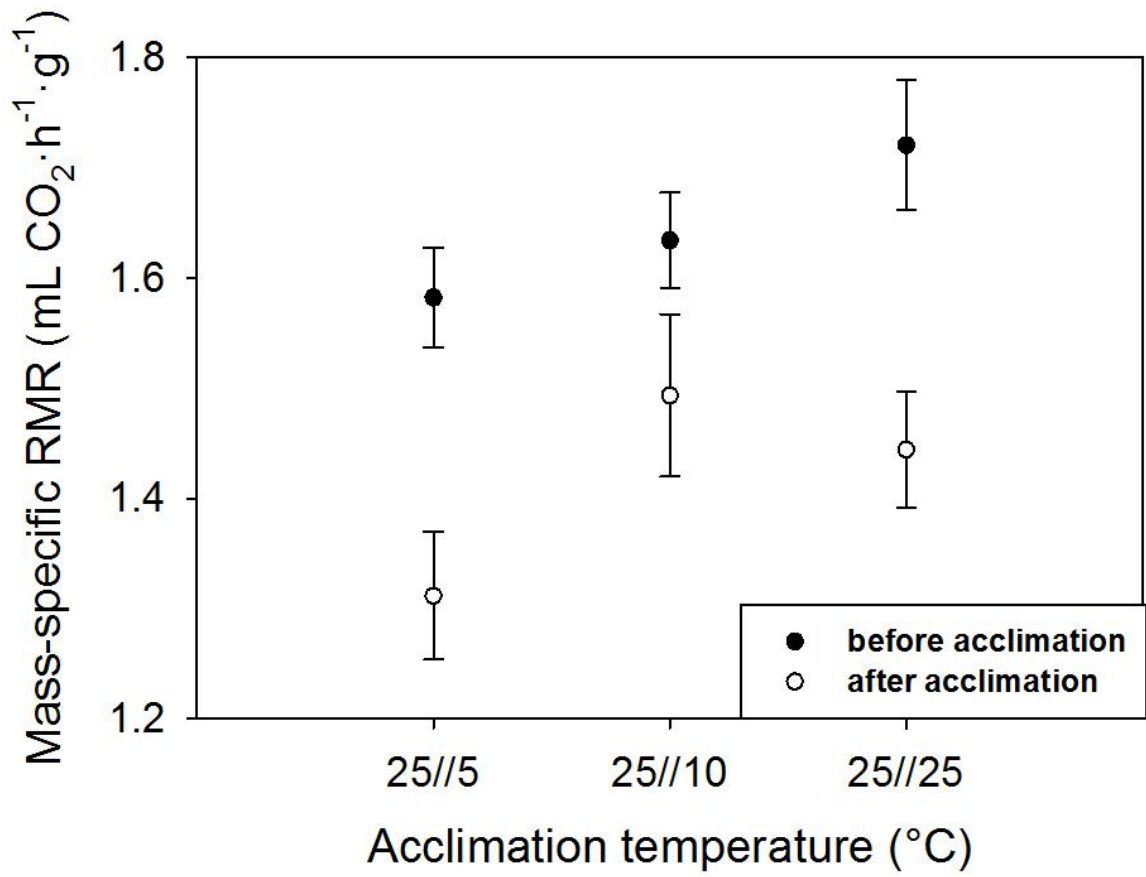


Figure 6. Mean mass-specific resting metabolic rate (RMR) \pm SE ($\text{mL CO}_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1}$) before and after acclimation at three different acclimation temperatures. Sample size varies from 47 to 88 worker bees for each group. Data are presented as mass-specific for visual presentation, but data were analyzed using whole-animal RestingMR with body mass as a covariate. Only bees acclimated to the control temperature (25//25) showed a significantly lower RestingMR post-acclimation compare to pre-acclimation measurements ($p=0.004$). Post-hoc tests revealed no effect of acclimation on RestingMR. See Results for details.

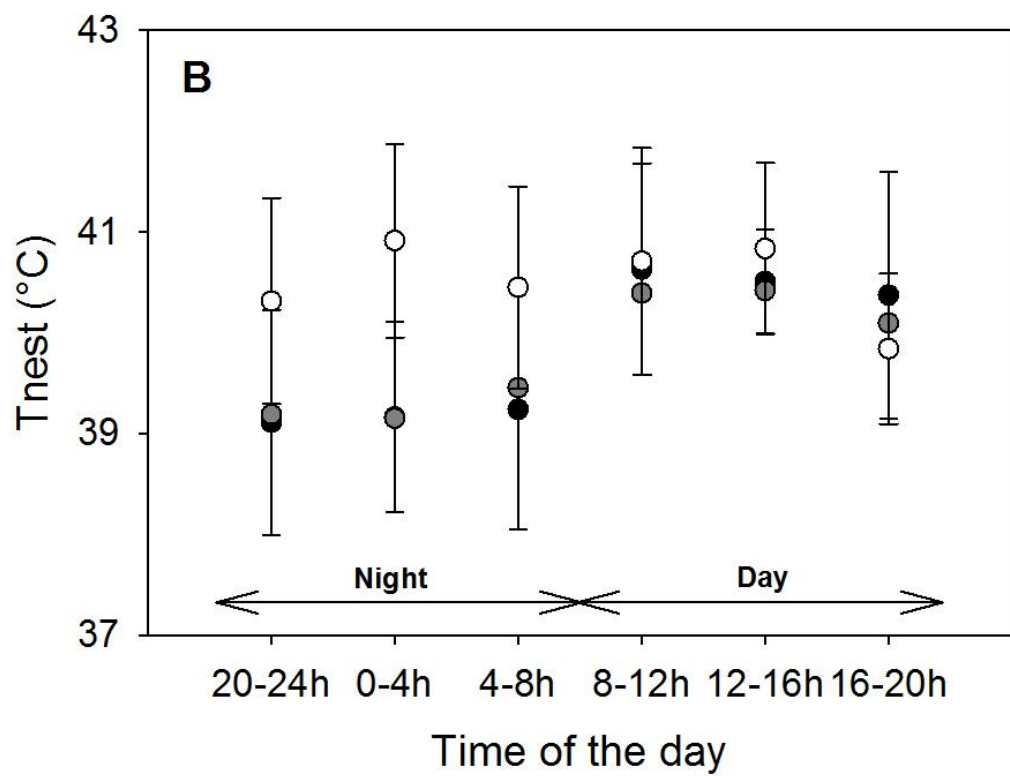
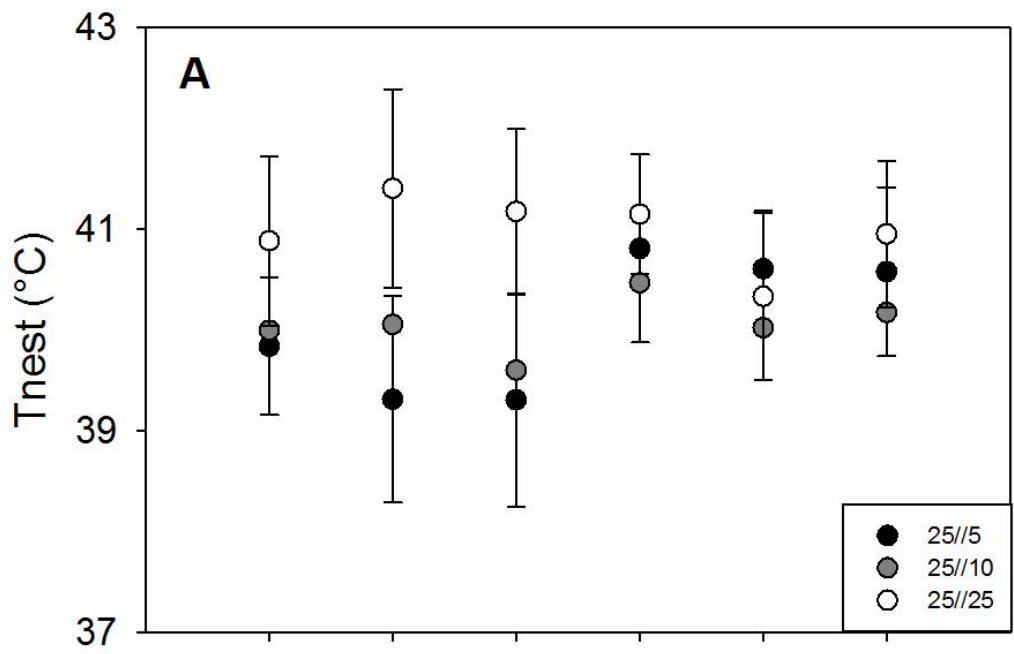


Figure 7. Mean maximum nest temperature ($T_{\text{nest}} \pm \text{SD}$ ($^{\circ}\text{C}$) during 24h before (panel A) and after acclimation (panel B) at three acclimation temperatures. A 12h:12h light cycle was used, where daytime started at 8h. The difference between the maximum nest temperature before and after acclimation was similar between the three acclimated groups, including the 25//25 control group. See Material and Methods and Results sections for details

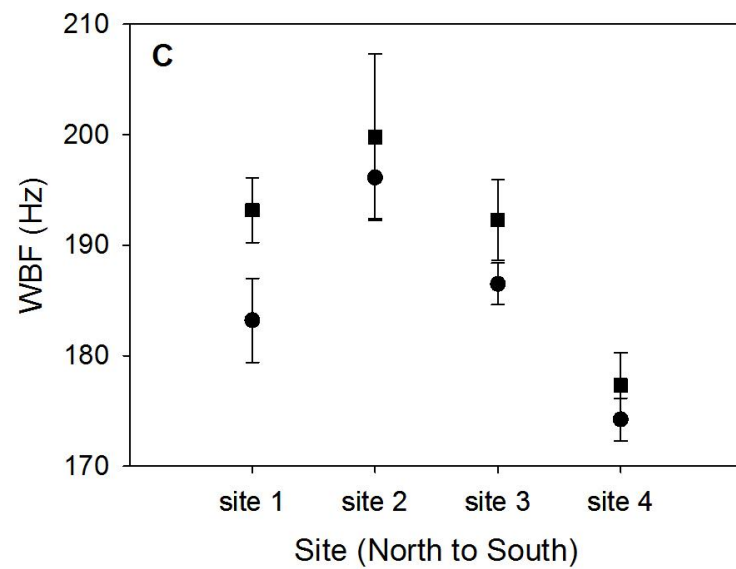
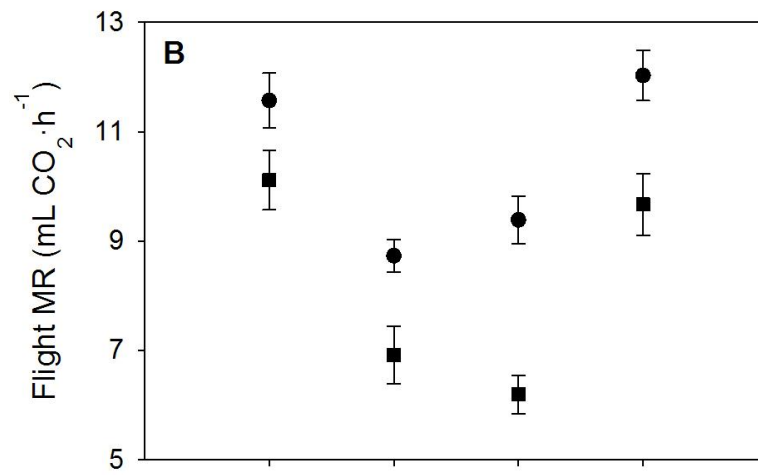
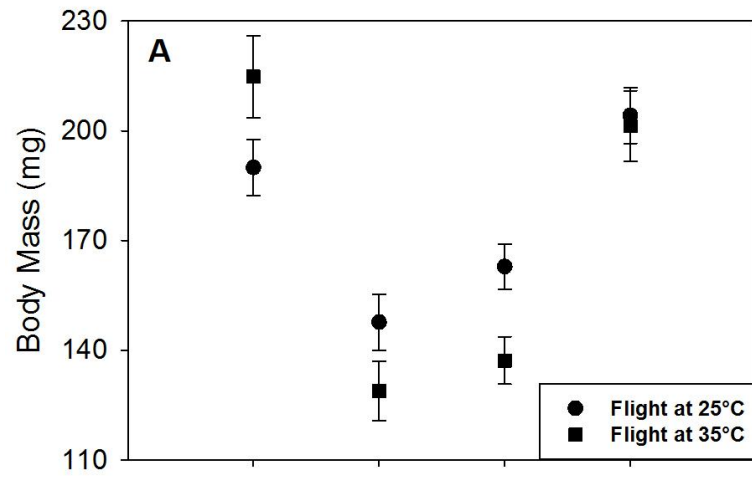


Figure 8. Mean \pm SE body mass (mg) (panel A), flight metabolic rate (FlightMR) (mL CO₂·h⁻¹) (panel B), and wingbeat frequency (WBF) (Hz) (panel C) in *B. impatiens* populations sampled at four latitudes, from North to South, and measured at two flight trial temperatures. Flight trial temperature has an effect ($p < 0.0001$) at sites 1, 3 and 4. Body mass varied among sites ($p = 0.001$), but neither FlightMR nor WBF varied among sites after accounting for body mass. (see Results for more details). Sample sizes were 15 to 44 worker bees per group for body mass and FlightMR, and 10 to 37 bees per group for WBF.

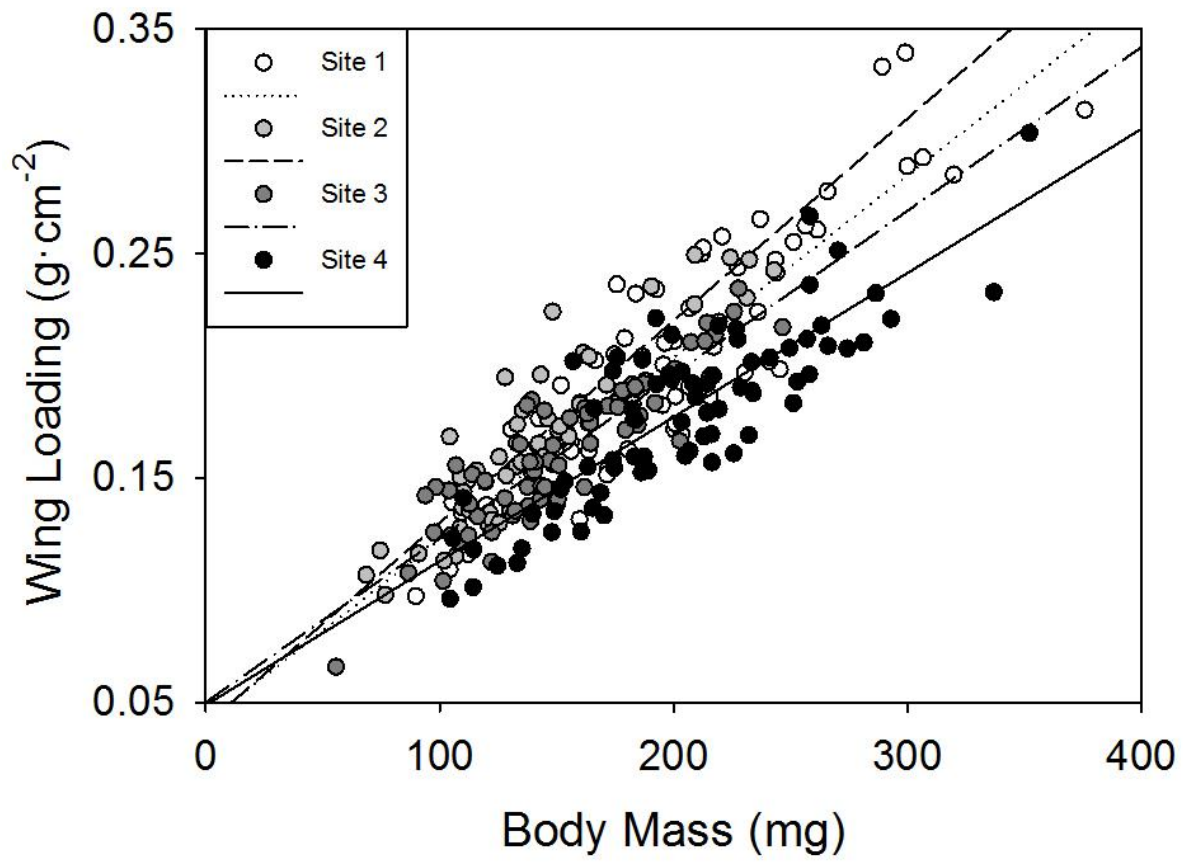


Figure 9. Relationship between wingloading ($\text{g}\cdot\text{cm}^{-2}$) and body mass (mg) in *B. impatiens* individuals from populations sampled at four geographical locations (from North to South, respectively site 1 to site 4). Significantly different slopes are observed between site 2 ($0.000901\text{mass}+0.039954$, $R^2=0.825$) and site 3 ($0.000730\text{mass}+0.049947$, $R^2=0.787$), site 2 and site 4 ($0.000642\text{mass}+0.048673$, $R^2=0.705$), and site 1 ($0.000812\text{mass}+0.041089$, $R^2=0.756$) and site 4. Sample size varied from 52 to 75 worker bees per site.

A



B



Figure 10. Representative images of night gathering (B) in 25//5 cold-acclimated colonies compared to daytime (A). At night, maximum nest temperature was constantly maintained above 36.8°C despite a surrounding temperature of 5°C. See Material and Methods for data collection.

Table 1. Summary of statistical results. NS means the variable had no statistical effect ($p>0.005$). Partial R^2 are indicated where a statistical effect was detected. temp= temperature, accl= acclimation temperature, mass=body mass, FlightMR= flight metabolic rate, RestingMR= RMR= resting metabolic rate, WBF=wingbeat frequency, “-“ = not applicable: the effect of this variable could not be tested for the respective dependant variable. (See Results for more details).

| | Dependant variable | total R² | flight temp. | acclimation temp. | acclimation state | site | body mass | time period | significant interactions |
|------------------------------|---------------------------|----------------------------|---------------------|--------------------------|--------------------------|-------------|------------------|--------------------|---------------------------------|
| 1st cohort | FlightMR | 0.565 | 0.340 | 0.010 | 0.060 | - | 0.013 | - | accl*mass 0.008 |
| | WBF | 0.470 | NS | NS | 0.035 | - | 0.336 | - | state*mass 0.022 |
| | RestingMR | 0.243 | - | 0.027 | 0.036 | - | 0.196 | - | accl*mass 0.027 |
| | RMR success | 0.524 | - | 0.305 | 0.219 | - | - | - | |
| 2nd cohort | FlightMR | 0.354 | NS | NS | NS | - | 0.354 | - | |
| | WBF | 0.231 | NS | NS | NS | - | 0.231 | - | |
| | RestingMR | 0.284 | - | 0.045 | NS | - | 0.120 | - | |
| 9week | body mass | 0.087 | - | NS | 0.087 | - | - | - | |
| | wing area | 0.919 | - | NS | NS | - | 0.919 | - | |
| | wing loading | 0.577 | - | NS | NS | - | 0.577 | - | |
| field | FlightMR | 0.570 | 0.110 | - | - | NS | 0.200 | - | site*mass 0.030 |
| | WBF | 0.459 | NS | - | - | 0.078 | 0.230 | - | |
| | body mass | 0.250 | - | - | - | 0.250 | - | - | |
| | wing area | 0.559 | - | - | - | 0.194 | 0.154 | - | |
| | wing loading | 0.796 | - | - | - | NS | 0.610 | - | site*mass 0.090 |

Table 2. Average (mean), sample size (n), and standard error (SE) of body mass, wingbeat frequency (WBF), flight metabolic rate (FlightMR), and resting metabolic rate (RMR) in 1-week old and 3-week old 2nd cohort worker bees acclimated since their development stage to 25°C (25//25) or 10°C at night (25//10).

| | acclimation temperature | 1-week old | | | 3-week old | | |
|------------------|-------------------------|------------|--------|------|------------|--------|-------|
| | | n | mean | SE | n | mean | SE |
| Body Mass | 25//10 | 18 | 189.37 | 7.26 | 20 | 212.26 | 11.51 |
| | 25//25 | 22 | 169.89 | 7.66 | 13 | 168.98 | 7.70 |
| WBF | 25//10 | 17 | 187.21 | 5.54 | 19 | 196.11 | 4.25 |
| | 25//25 | 22 | 195.26 | 2.91 | 13 | 193.96 | 2.62 |
| FlightMR | 25//10 | 18 | 10.32 | 0.88 | 20 | 12.65 | 0.90 |
| | 25//25 | 22 | 9.30 | 0.51 | 13 | 9.58 | 0.51 |
| RestingMR | 25//10 | 15 | 0.32 | 0.02 | 17 | 0.38 | 0.06 |
| | 25//25 | 23 | 0.26 | 0.01 | 13 | 0.21 | 0.02 |

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