Matters of Size: Behavioral, Morphological, and Physiological Performance Scaling Among Stingless Bees (Meliponini)

by

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ABSTRACT

Body size plays a pervasive role in determining physiological and behavioral performance across animals. It is generally thought that smaller animals are limited in performance measures compared to larger animals; yet, the vast majority of animals on earth are small and evolutionary trends like miniaturization occur in every animal clade. Therefore, there must be some evolutionary advantages to being small and/or compensatory mechanisms that allow small animals to compete with larger species. In this dissertation I specifically explore the scaling of flight performance (flight metabolic rate, wing beat frequency, load-carrying capacity) and learning behaviors (visual differentiation visual Y-maze learning) across stingless bee species that vary by three orders of magnitude in body size. I also test whether eye morphology and calculated visual acuity match visual differentiation and learning abilities using honeybees and stingless bees. In order to determine what morphological and physiological factors contribute to scaling of these performance parameters I measure the scaling of head, thorax, and abdomen mass, wing size, brain size, and eye size. I find that small stingless bee species are not limited in visual learning compared to larger species, and even have some energetic advantages in flight. These insights are essential to understanding how small size evolved repeatedly in all animal clades and why it persists. Finally, I test flight performance across stingless bee species while varying temperature in accordance with thermal changes that are predicted with climate change. I find that thermal performance curves varied greatly among species, that smaller species conform closely to air temperature, and that larger bees may be better equipped to cope with rising temperatures due to more frequent exposure to high temperatures. This information may help us

predict whether small or large species might fare better in future thermal climate conditions, and which body-size related traits might be expected to evolve.

DEDICATION

I dedicate this document to my husband, family, and close friends who have helped me to grow and succeed through their endless support, and to anyone striving to achieve while battling a chronic illness.

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PREFACE

The importance of animal body size in biology

There is colossal variation in body size among animals; blue whales are more than 760 billion times more massive than the smallest animals (Polilov 2015, Milo et al. 2010). Body size has a profound and pervasive effect on every aspect of animal physiology and behavior (Peters 1983, Hanken & Wake 1993, Eberhard & Wcislo 2011, Bonner 1979). Generally, it is thought that bigger is better. Larger animals have advantages in locomotion (longer strides, faster movements, greater power per limb stroke), foraging (able to eat more food faster, and able to eat food with lower nutrition, can move greater distances to find food sources), defensive and aggressive behaviors (are more often able to be successful predators, usually win in mating competitions, usually win territorial disputes [Bonner 1979, Peters 1983]). Larger animals also have lower mass-specific metabolic rates enabling them to do more with less energy per gram of their body mass (Brown et al. 2004, West 2002, Hulber & Else 2000).

Small animals are thought to be disadvantaged in these categories for a number of reasons. Smallness limits the amount of space available in the body for tissues of all types (Hanken & Wake 1993, Eberhard & Wcislo 2011). This must result in reductions, structural simplification, novel traits, and/or increased variability in morphological characters (Hanken & Wake 1993). Higher mass-specific energy use in smaller animals confers much greater costs on all activities. Moving the same distance as a larger animal requires a greater number of limb cycles, whether walking, swimming or flying (Morales & Ellner 2002). Small animals maintain smaller territories and cannot usually move as far in search of mates or food (Reiss 1988). Smaller animals more often have specialized

diets, limiting the possible items they might forage on. They are more likely to be prey for larger animals, even if they are also predators of smaller organisms. Because they have shorter limbs and less power in movement, smaller animals generally lose in tests of strength during territorial and mating competition (Foot 1988, Hunt et al. 2009).

Predominance of small animal species

Despite these disadvantages, small body size is present in every animal clade, and much more common than large size (Blackburn 1994) suggesting that there must be some selective advantages to being small (Hanken & Wake 1993). Miniaturization, the evolution of smaller body size compared to ancestral species or generations, is an example of small size that further suggests tininess can be advantageous (Hanken & Wake 1993). Some of the best-documented examples include salamanders, parasitic wasps (Polilov 2012), featherwing beetles (Polilov 2008), and many immature and larval forms. However, there are examples of miniaturized castes within social species, such as ant minims (Poulson 2010) and tiny stingless bee queens (Ribeiro et al 2006).

It is unclear if small animals have behavioral or physiological distinctions from larger animals. Possibly, small animals adopt different or compensatory behavioral strategies and/or have alternate or compensatory physiological mechanisms for completing tasks. Small and/or miniaturized animals often have exaggerated features such as ornamentation (also found in larger animals) or proportionally larger body parts, such as larger brains, heads, and sensory structures relative to body mass (Rensch 1948). Rensch's rule also shows that in smaller species, females of dimorphic species often tend to be larger and able to carry a proportionally larger number of eggs or offspring than

larger animals. Males of smaller species are less likely to develop exaggerated traits, saving a large amount of energy in the development and maintenance of such traits (Rensch 1950, Abouheif & Fairbairn 1997).

The effects of small body size on behavior

With smaller overall brain size (absolute size), small animals are thought to have smaller behavioral repertoires, less complex behaviors, and lesser learning capabilities. Within and among ant species, bigger brains are associated with generalized worker castes. Ants with more specialized tasks tend to have smaller brains, perhaps because specialization does not require them to maintain the brain tissue needed for a wider range of behaviors (Whener at al. 2007, Cole 1985, Gronenberg & Riveros 2009). Similarly, in other insects and fish, bigger brains are correlated with generalist feeding strategies (Hahn et al. 2012, Harvey et al. 1980, Schoenemann 2004, Farris & Roberts 2005). Marsupials with larger brains have greater limb dexterity for precise behaviors (Iwaniuk et al. 2000). Within bumblebee species, larger workers with bigger brains learn foraging tasks more quickly (Iwaniuk et al. 2000). Among apes, humans have the largest brain relative to body size and we have substantially larger behavioral repertoires and cognitive abilities than chimpanzees, bonobos, and other related apes (Herculano-Houzel 2009, Gibson et al. 2001) and overall brain size is a good predictor of cognitive abilities (Deaner et al. 2007). Recently, researchers have even found gene differences between humans and chimpanzees that result in 12% larger brain size and greater cognitive abilities when introduced into mice (Boyd et al. 2015).

However, there is mounting evidence that absolute brain size and brain size relative to body mass may not be good predictors of behavioral abilities. Among rodent and salamanders studied, overall brain size does not correlate with behavioral repertoire size or the ability to carry out certain specific behaviors to the same level of competency (Campi & Krubitzer 2010, Roth et al. 1995, Miklos 1998). Again, none of these studies have attempted to explicitly examine the effect of body size on brain size and/or function within a clade of animals.

As a rule, smaller animals have proportionally larger heads and brains relative to their body size (Haller's rule [Rensch 1948]). This may allow them to maintain a greater number of brain functions, ultimately improving behavioral repertoires and abilities. In studies of tiny featherwing beetles and parasitoid wasps, smaller body size correlated with smaller and fewer neurons (Polilov 2008, Polilov 2012, Chittka & Niven 2009). The remaining brain tends to be organized and complex despite reduction in size (Niven 2010, Niven & Farris 2012, Kaas 2000). It has been suggested that large brains have built-in redundancy (more neuronal pathways than required for a task, more cortical modules than needed, etc. [Anderson 2010, Tononi et al. 1994]). Also, there is some evidence that tiny parasitoid wasps may lyse the nuclei of their neurons in development in order to achieve smaller neurons (Polilov 2012). This suggests that structural changes (rather than brain size) in small brains can accommodate maintenance of behavioral capabilities (Cuntz et al. 2013, Gonzalez et al. 2013), but there is no consensus on whether this applies across animals.

Challenges of studying small animals

While there are many scaling studies of metabolic rate, locomotion, foraging, mating, learning, etc., the vast majority are done on larger animals in all clades. Often, scientific instruments are not built for precision in measuring tiny organisms or tiny quantities on the scale of miniaturized animals. Additionally, little is often known about the basic ecology and natural history of small and miniaturized animals that are in the background compared to larger, more obvious animals. Despite this, there are excellent reasons to study how and why so many animals are small. It is vital for understanding of how body size impacts physiology and behavior in all animals, how and why there is diversity in body size, and what evolutionary pressures differentially affect animals of different sizes. Additional practical applications include improving the design of miniature computing and flying devices, and even simplifying machinery and programming code for energetic efficiency.

While behavior and physiology are greatly impacted by body size, body size is also important for determining how organisms interact with physical environmental variables. The way in which an organism regulates and responds to temperature plays an especially large role in determining that organism's success. As body size decreases, the role of ambient temperature in physiological performance increases tremendously. For example, small ectotherms warm up and cool down faster, tracking ambient temperatures closely, because of large body surface area to volume ratios (May 1979, Stevenson 1985). Larger ectotherms can often thermoregulate while smaller ones cannot. Bumblebees, for example, can warm themselves on cool mornings by shivering their flight muscles until they reach a temperature that allows them to fly (Heinrich & Esch 1994). Smaller

ectotherms must wait until their bodies reach a temperature suitable for activity, perhaps limiting the time they can be active (Stevenson 1985, Pereboom & Beismeier 2003). Ectotherms of all sizes use behavioral thermoregulation such as shade-seeking behaviors and some use evaporative cooling when necessary (Weiss & Laties 1961). The global distribution of ectotherms suggests that larger ectotherms are more suited to living in colder environments and closer to the poles, whereas there is huge diversity of smaller ectotherms closer to the equator (Huey & Kingsolver 1989, Dillon et al. 2010). This suggests that smaller species may have an advantage in hotter conditions. However, the scaling of thermal tolerance and the full effects of temperature on behavior and physiology are unknown.

Stingless bees as a model system for studying behavioral and physiological scaling

Stingless bees (tribe Meliponini) are an ideal group to study behavioral and physiological scaling and the evolution of small body size. Throughout the clade, there is a three orders of magnitude range of body mass with multiple small and miniaturized lineages (Roubik 1989, Camargo 2013, Michener 2000, Rasmussen & Cameron 2009). Though the body size range among stingless bee species is very small compared to the overall range of body size differences among all animals, it is useful because of the distribution of body size differences across the stingless bee phylogeny. Small body size is not simply dependent upon relatedness in this group but has arisen multiple times across the clade. Ancestral reconstructions based on relatedness to other bee groups (honey bees, bumble bees, and orchid bees) shows that the common ancestor to stingless bees was likely medium sized, probably around 50 mg (Rasmussen & Cameron 2009),

implying that some many species have miniaturized relative to their common ancestor and some have increased in size. The extant phylogeny of this group is fairly complete, allowing for phylogenetically controlled comparisons among species. Stingless bees are found throughout the world's tropical regions (Michener 2000, Camargo 2013). In many areas of South and Central America, Africa, and Asia, they are the most numerous and diverse bees. In total, over 500 species have been identified (Michener 2000). Stingless bees are important tropical pollinators, with one species pollinating upwards of 100 different trees, lianas, shrubs, crops, and other flowering plants (Roubik 1989, Roubik 2000). These ecosystem services make them an important group for study.

Preview of dissertation chapters

To date, few phylogenetically controlled comparative studies of cognitive behavioral scaling exist. Many have compared the abilities of various life stages or social castes within the same species (Cole 1985, Eberhard & Wcislo 2011, Eberhard 2007, Dial et al. 2008) and have found a variety of results. In some cases, younger larval or nymph stages are adapted to completely different behaviors than adults, such as the aquatic larvae of amphibians (Hanken & Wake 1993). Individuals in different morphological castes, such as those in highly social ant species, can also have very different morphologies, behaviors, and even underlying neurophysiologies such as structural and chemical brain pathways (Wilson 1978, Zube & Rossler 2008). Studying the effects of body size on behavioral and physiological functions across species is challenged by difficulties in finding behaviors and functions that are clearly analogous. If small and

large species have very different life histories, feeding strategies or sensory biologies, it can be difficult or impossible to clearly distinguish effects of body size

In this dissertation, I compare the scaling of behavioral performance and costs in visual learning and flight among stingless bee species. Both flight and learning behaviors are necessary for foraging and other resource collection, dispersal and migration, mating displays, and defensive behaviors in flying insects, birds, and bats. These behaviors have been well-documented in vertebrate and invertebrate animals including birds, bats, and insects. However, no scaling studies exist to clarify the consequences of small body size in visual learning or flight in either performance or cost.

First, I develop a behavioral test of visual acuity models based on eye morphology using honey bees. Visual acuity has been measured in honeybees and they are well known to do a number of visual learning tasks. I used a y-maze discrimination test to determine whether honeybee visual discrimination performance matches measurements of visual acuity. I then adapted this test in order to compare stingless bee visual discrimination performance across body size. This is the first phylogenetically and ecologically controlled comparative study of the effect of body size on learning rates in any taxonomic group. I use the Y-maze discrimination test adapted from the honeybee study and added a learning assay to determine whether bee species of different sizes can learn associations among patterns they can resolve. Then, I aim to examine the scaling of visual acuity across body size among stingless bee species. I measure eye morphology to understand any tradeoffs in acuity at small body sizes and then test whether visual learning abilities are limited by size in smaller species. I will also measure head and brain size in order to determine whether stingless bees follow Haller's rule. This will help

determine whether Haller's rule aids in compensating for small size issues. Establishing whether smaller animals are cognitively limited in learning tasks, the role of sensory structure and brain size in those tasks, and how learning behaviors are different among small and large species is necessary for learning how and why small size is so pervasive among animals because behavioral performance is likely to be a major target of natural selection.

Studies on flying insects, bats and birds suggest hypometric scaling of flight metabolic rate occurs across groups (Bartholomew & Casey 1978, Marden 1994, Nicen & Scharleman 2005). However, in insects, these studies used larger animals and very small insects seem to have unusually low flight metabolic rates. We do not understand why hypometric scaling occurs, and this raises the question of whether the scaling of flight MR differs as fliers move into smaller sizes and why. I aim to quantify flight metabolic rates, wing beat frequencies, and load-carrying abilities among stingless bee species. To define which morphological and/or physiological mechanisms underlie any differences from typical scaling patterns, I will also measure morphological traits such as wing, head, thorax, and abdomen size to show how these traits might determine flight performance, especially in smaller species. This is important for understanding the energetic and structural tradeoffs of flight across different size ranges.

Finally, I aim to shed light on how increases in environmental air temperature might affect flight performance across stingless bee species. There is a great deal of information on how animals of different sizes gain and shed heat, produce heat, behaviorally thermoregulate, and how small and large species are distributed across different temperature zones; I will test how flight metabolic rate scales across size and

temperature in stingless bees. This will enable me to speculate on whether small or large species will have advantages in the hotter temperatures predicted by climate change.

Significance

These insights into the scaling of flight, learning, and thermal performance among stingless bees will shed light on how small species manage to compete with larger animals. Smaller species may have compensatory mechanisms or alternate strategies. Further, the data contained in this dissertation are important for understanding why the vast majority of animals are small, as well as how and why miniaturized species have repeatedly evolved across the animal kingdom. Looking forward, these data may be important for predicting how warmer temperatures will might differentially affect the performance of small and large animals, whether small or large animals will be better equipped to deal with climate change, and what types (morphological, physiological, and behavioral) body size-linked traits might be expected to evolve in the projected warmer global climate.

REFERENCES

Abouheif E., Fairbairn D. J. 1997. A comparative analysis of allometry for sexual size dimorphism: assessing Rensch's rule. *Am. Nat.* 149(3)- 540-562.

Abramowski D, Currie C.R, Poulson M. 2011. Caste specialization in behavioral defenses against fungus garden parasites in Acromyrmex octospinosus leaf-cutting ants. *Insectes Sociaux* 58: 65-75.

Anderson, M. L. 2010 Neural reuse: a fundamental organizational principle of the brain. *Behav. Brain Sci.* 33, 245–313.

Barton, R.A., Harvey, P.H. 2000. Mosaic evolution of brain structure in mammals. *Nature* 405, 1055-1058.

Blackburn, T. M. 1994. Animal body size distributions: patterns, mechanisms and implications. *TREE* **9**(12): 471-474.

Bonner, J. 1979. *Why Size Matters.* (Princeton University Press, Princeton, NJ. (2006).

Boyd, J.L., Skove, S.L., Rouanet, J.P., Pilaz, L., Bepler, T., Gordan, R., Wray, G.A., Silver, D.L. 2015. Human-chimpanzee differences in a FZD8 enhancer alter cell cycle dynamics in the developing neocortex. *Current Biol.* 25(6): 772–779.

Bozinovic, F., Bastias, D., Boher, F., Clavijo-Baquet M., Estay, S.A., Angilletta, M.J. 2011. The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiol Biochem. Zool. 84* (6): 543-552.

Camargo, J.M.F. 2013. Historical biogeography of the Meliponini (Hymenoptera, Apidae, Apinae) of the Neotropical Region. *Pot Honey: a legacy of stingless bees.* 19-34 (P. Vit, S.R.M. Pedro, and D.W. Roubik. Springer Science + Business, New York).

Chittka, L. and Niven, J. 2009. Are bigger brains better? *Curr. Biol.* 19, 995–1008.

Cole, B. J. 1985. Size and behavior in ants: constraints on complexity. *Proc. Natl. Acad. Sci.* USA 82, 8548–8551.

Cuntz, H., Forstner, F., Schnell, B., Ammer, G., Raghu, S.V., Borst, A. 2013. Preserving neural function under extreme scaling. *PloS one* 8:8, e71540.

Development and Evolution of Brain Size: Behavioral Implications. Edited by Hahn, M.E. et al. Academic Press Inc., New York. 2012.

Dial, K. P., E. Greene and D. J. Irschick. 2008. Allometry of behavior. *Trends in Ecol. Evol.* 23(7): 394-401.

Dillon ME, Wang G, Huey RB. 2010. Global metabolic impacts of recent climate warming. *Nature*. 467:704-707.

Eberhard W. G. 2007. Miniaturized orb-weaving spiders: behavioural precision is not limited by small size. *Proc Biol Sci.* 274(1622): 2203–2209.

Eberhard, W., Wcislo, W. 2011. Grade changes in brain-body allometry: morphological and behavioural correlates of brain size in miniature spiders, insects and other invertebrates. *Adv. Insect Physiol.* 40, 155-213.

Farris, S., Roberts, N. 2005. Coevolution of generalist feeding ecologies and gyrencephalic mushroom bodies in insects. *Proc. Natl. Acad. Sci.* 102, 17394-

17399.

Felsenstein, J. 1985. Phylogenies and the comparative method. *Am. Nat.* 125, 1–15.

Foote CJ. 1988. Male Mate Choice Dependent On Male Size in Salmon. *Behaviour* 106(1): 63-80.

Garamszegi, L.Z. 2014. *Modern Phylogenetic Comparative Methods and Their Application in Evolutionary Biology*. Springer-Verlag Berlin Heidelberg, Germany.

Gibson, Kathleen R., Duane Rumbaugh, and Beran, M. 2001. Bigger is better: primate brain size in relationship to cognition." *Evolutionary anatomy of the primate cerebral cortex*: 79-97.

Gillooly, J., Brown, J., West, G.B., Savage, V.M., Charnov, E.L. 2001. Effects of size and temperature on metabolic rate. *Science.* 293: 2248-2251.

Gonzalez, L.A., Benefit, B.R., McCrossin, M.L., Spor, F. 2015. Cerebral complexity preceded enlarged brain size and reduced olfactory bulbs in Old World monkeys. *Nature Comm.* 6:7580 DOI 10.1038/ncomms8580.

Gronenberg, W. and Riveros, A. J. 2009. Social brains and behavior-past and present. In: Organization of Insect Societies (eds Gadau, J. and Fewell, J.), pp. 377–401. Harvard University Press, Cambridge.

Hanken, J. and Wake, D. B. 1993. Miniaturization of body size: organismal consequences and evolutionary significance. *Ann. Rev. Ecol. Syst*. 24, 501–519.

Harvey, P. H., Clutton-Brock, T. H. and Mace, G. M. 1980. Brain size and ecology in small mammals and primates. *Proc. Natl. Acad. Sci.* USA 77, 4387–4389.

Harvey, P. H., Pagel, M.D. 1991. *The Comparative Method in Evolutionary Biology.* (Oxford University Press, New York, NY).

Herculano-Houzel, S Mota, B., Lent, R. 2006. Cellular scaling rules for rodents. *Proc. Natl. Acad. Sci.* 103(32): 12138-12143.

Herculano-Houzel, S. 2009. The human brain in numbers: a linearly scaled up primate brain. *Frontiers Neurosci* 3(31): 1-11.

Huey R. & Kingsolver J. 1989. Evolution of thermal sensitivity of ectotherm performance. *Trends Ecol. Evol*. 4(5): 131-135.

Hulbert AJ and Else PL. 2000. Mechanisms underlying the costs of living animals. *Annu. Rev. Physiol.* 62: 207-235.

Hunt K, Breuker CJ, Sadowski JA, Moore AJ. 2009. Male–male competition, female mate choice and their interaction: determining total sexual selection. *J Evol Biol.* 22(1): 13-26.

Invertebrate Learning and Memory. Menzel R, Benjamin P.R. Academic Press, Elsevier 2013.

Iwaniuk, A. N., Nelson, J. E. and Whishaw, I. Q. 2000. The relationships between brain regions and forelimb dexterity in marsupials (Marsupialia): a comparative test of the principle of proper mass. *Aust. J. Zoo.* 48, 99–110.

Kaas, J. 2000. Why is brain size so important: design problems and solutions as neocortex gets bigger or smaller. *Brain Mind* 1:7-23

Kingsolver, J. and Huey, R. 2008. Size, temperature, and fitness: three rules. *Evol. Ecol. Research* 10: 251-268.

Kozłowski, J. and A. T. Gawelczyk (2002). "Why are species' body size distributions usually skewed to the right?" *Functional Ecology* **16**(4): 419-432.

Marden J. H. 1994. From damselflies to pterosaurs: How burst and sustainable flight performance scale with size *Am. J. Physiol.* DOI: 10.1152/ajpregu.1994.266.4.R1077

Michener, C. D. 2000. *The Bees of the World*. Johns Hopkins University Press. Baltimore, Maryland, United States.

Miklos, G. L. G. (1998). The evolution and modification of brains and sensory systems. *Daedalus* 127, 197–216.

Milo, R, Jorgensen, P, Moran, U, Weber, G, Springer M. 2010. BioNumbers- the database of key numbers in molecular and cell biology. *Nucleic Acids Research* 38: D750–D753.

Morales J. M, Ellner SP. 2002 Scaling up animal movements in heterogeneous landscapes: the importance of behavior. *Ecology* 83(8): 2240-2247

Niven J. E., Scharlemann J. P. W. 2005. Do insect metabolic rates at rest and during flight scale with body mass? *Biol Lett.* 1(3): 346–349.

Niven, J. E. 2010. Nervous system evolution in relation to behavior. *Encyclopedia of Animal Behavior 2*, 527–533 (Oxford Academic Press, New York).

Niven, J., Farris, S. 2012. Miniaturization of nervous systems and neurons. *Current Biol.* 22, R323-R329.

Niven, J.E., Scharlemann, J.P.W. 2005. Do insect metabolic rates at rest and during flight scale with body mass? *Biol. Lett.* 1: 346–349.

Pereboom, J.J.M., Biesmeijer, J.C. 2003. Thermal constraints for stingless bee foragers: the importance of body size and coloration. *Oecologia*. 137(1): 42-50.

Peters, R.H. 1983.*The Ecological Implications of Body Size*. (Cambridge University Press, New York).

Polilov, A. 2015. Small Is Beautiful: Features of the Smallest Insects and Limits to Miniaturization. *Annu. Rev. Entomol.* 6:103-121.

Polilov, A. A. 2008. Anatomy of the smallest Coleoptera, featherwing beetles of the tribe Nanosellini (Coleoptera, Ptiliidae), and limits of insect miniaturization. *Entomol. Rev.* 88, 26–33.

Polilov, A. A. 2012. The smallest insects evolve anucleate neurons. *Arthropod. Struct. Dev.* 41, 29–34.

Rasmussen, C. and Cameron, S. A.2009. Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. *Biol. J. Linn. Soc.* 99, 206–232.

Reiss M. 1988. Scaling of home range size: Body size, metabolic needs and ecology. *Cell Commentary.*

Rensch B. 1950 Die Abhangigkeit der relative sexualdifferenz von der Korpergroβe. *Bonner Zoologische Beiträge* 1:58-69

Ribeiro M. F, Wenseleers T, Santos Filho P.S., Alves D.A. 2006. Miniature queens in stingless bees: basic facts and evolutionary hypotheses. *Apidologie* 37: 191-206.

Roth, B.G., · Blanke, J., · Ohle, M. 1995. Brain Size and Morphology in Miniaturized Plethodontid Salamanders. *Brain Behav. Evol.* 45:84–95.

Roubik D.W. 1989. *Ecology and Natural History of Tropical Bees.* Cambridge University Press, Cambridge, United Kingdom.

Roubik, D.W. 2000. Pollination system stability in Tropical America. *Conservation Biology.* 14 (5): 1235-1236.

Schoenemann, P. T. 2004. Brain size, scaling and body composition in mammals. *Brain Behav. Evol.* 63, 47–60.

Stone G. N, Willmer P. G. 1989. Warm-up rates and body temperatures in bees: the importance of boy size, thermal regime, and phylogeny. *J. Exp. Biol.* 147: 303-328.

Tononi, G., Sporns, O. and Edelman, G. M. 1994. A measure for brain complexity: relating functional segregation and integration in the nervous system. *Proc. Natl. Acad. Sci.* USA 91, 5033–5037.

Wehner, R., Fukushi, T. and Isler, K. 2007. On being small: brain allometry in ants. *Brain Behav. Evol*. 69, 220–228.

Weiss, Laties. 1961. Behavioral thermoregulation. *Science.* 133(3461): 1338-1344.

West, G.B. 2002. Allometric scaling of metabolic rate from molecules and mitochondria to cells and mammals. *Proc. Natl. Acad Sci.* 99, 2473-2478.

Wilson, E.O. 1978. Division of labor in fire ants based on physical castes (Hymenoptera: Formicidae: *Solenopsis*). *J. Kansas Entomol. Soc.* 51(4): 615-636.

Zube C., Rossler W. 2008. Caste- and sex-specific adaptations within the olfactory pathway in the brain of the ant Ca*mponotus floridanus*. *Arthtropod Struc. Devel.* 37(6): 469-479.

CHAPTER 1

A BEHAVIORAL ASSESSMENT OF HONEY BEE SPATIAL RESOLUTION: PATTERN DIFFERENTIATION AND LEARNING ABILITIES MATCH EYE MORPHOLOGY

ABSTRACT

The ability to distinguish patterns is important for insects that must navigate a visually complex world. Many insects rely on visual cues to successfully navigate during foraging, migration, and other activities. For bees, flowers may consist of similar patterns, shapes and colors that must be distinguished and learned to obtain the best nectar and pollen food resources. The resolution and acuity of the honeybees' apposition compound eye have been calculated from ommatidial (facet) measurements but not tested behaviorally. In this study, I used a Y-maze to test the capacities of honeybees to learn to distinguish black and white visual patterns (one of which was paired with a sucrose reward) that varied in the spatial frequency of the pattern lines. Free-flying honeybee foragers did not have any innate preferences for the patterns. In the Y-maze test, honeybees had better vision than predicted by measurements of ommatidia. However, the ability of bees to learn pattern associations in the Y-maze was less than expected based on the differentiation test. The learning task may have been too difficult in some cases (when patterns were spatially similar) or too easy (when patterns were substantially different spatially, e.g. black vs. white).

INTRODUCTION

All insects must be able to see and recognize visual patterns across orders of magnitude in size and detail. This ability is vital to navigation, foraging, and many other behaviors. In a variety of insects, eye characteristics have been measured in order to estimate what they may be able to see at given distances. In some (butterflies, dung beetles, crepuscular bees, etc.), visual acuity and resolution (the ability to differentiate objects in the visual field as different) have been directly measured (Land & Nilsson 2012). This allows functional behavioral testing of the accuracy of those measurements and their predictions. Functional visual abilities and eye measurements do no always match, especially in cases where eye movements enhance vision or there are additional steps in neural processing which may improve vision, such as in neural superposition where sensitivity is improved by combining the input of multiple facets (Land & Nilsson 2012). These cases must be demonstrated through electrophysiology or microscopy, but there are few behavioral demonstrations of visual acuity (Land & Nilsson 2012).

The spatial resolution of the apposition compound eyes of honey bees have been estimated from the interommatidial angles for certain eye regions (frontal, upper, and lower regions), the sizes of ommatidia, and physiological characteristics of photoreceptors (Srinivasan 2010, Seidl & Kaiser 1981). These calculations suggest that the smallest detectable object for honey bees must fill at least seven ommatididia (eye facets) in order to be seen (Srinivasan 2010, Seidl & Kaiser 1981, Giurfa & Vorobyev 1998). Objects filling smaller than seven ommatidial fields of view would theoretically not be seen, and patterns with frequencies above seven ommatidial fields should appear

as a blur. The spatial frequency (number of pattern components within a given area of the visual field), contrast (the difference in visual properties among components), and intensity (the brightness of the components) of a floral pattern are important for identification by bees (Abramson et al. 2013, Giurfa et al. 1999a & b, Galizia et al. 2012). For example, if two objects, or lines in a pattern, are very close together, they may not be distinguishable as separate units. When an object in the visual field takes up a large portion (greater than 15º for honeybees) of the visual field, its features blur together based on lack of contrasting cues surrounding them in the honey bee visual field. When there are two similar objects close together, blurring also occurs (Srinivasan 2010).

As a highly useful model organism, the visual system and behavioral abilities of honey bees has been one of the best-studied among insects (reviewed in Menzel 2012, Srinivasan 2010, Galizia et al. 2012, Matthews & Matthews 2010). Honeybees can be readily trained to associate different colors and shapes with aversive or appetitive reinforcement (Avargués-Weber et al. 2012, Giurfa et al. 1999, Hempel de Ibarra et al. 2002, Benard & Giurfa 2008; reviewed in Srinivasan 2010, Land 1997, Land & Nilsson 2012, and others). Proboscis extension reflex conditioning has been used to teach bees a number of cues but can be restrictive and gives very little additional behavioral information (Galizia et al. 2012). Some studies have also used free-flying bees attracted to feeders and trained them to visual stimuli (Avargués- Weber et al. 2010).

In this study I put foraging honeybees through a carefully controlled Y-maze visual learning task (Zhang et al. 1996) to ask: (1) Does functional visual acuity match predictions based on the optical capabilities of honeybee compound eyes? And, how does

the ability to visually differentiate patterns relate to the capacity to associatively learn these patterns? I test the hypothesis that honeybees have a threshold spatial resolution and that the difference in spatial frequency between two black and white patterns will predict the difficulty of distinguishing between these patterns, and the capacity to learn that food rewards are associated with these patterns.

METHODS

Experiment 1: Testing for pre-existing preferences for patterns

In nature, bees learn to associate floral resources with particular visual cues so it was important to determine if honeybee foragers (*Apis mellifera*) had innate preferences for any of the experimental visual patterns I planned to use in the maze experiments. They were lured to feeders with 50% sucrose solution near Arizona State University Tempe Campus. Visual patterns (Table 1) were laid out on a table in two rows in random order, all with sucrose rewards. Each pattern was 12.7 cm in diameter and was placed on a15.25 cm transparent petri dish. Each pattern had a transparent 2.5 cm petri dish glued to the center. Sucrose rewards were placed in the smaller petri dish so bees could drink *ad libitum*. The pattern that each bee landed and drank from was recorded by taking photographs at ten-minute intervals for two hours. Only feeding bees (touching the sucrose reward with proboscis out) were counted because they made a clear choice of patterns to forage at, whereas flying or walking bees may not have committed to foraging at that pattern. To avoid influencing bees' decision in any way, bees were not marked or removed from the population once observed at a feeder. This could mean that some bees

were counted in more than one photograph sample on a given day. Observations were repeated on three separate days.

The average number of bees present at each pattern over the course of each two-hour observation period was calculated. On each day, the total number of bees differed, so data were analyzed as proportions. Data were unevenly distributed based on a Levene's test for variance and visual assessment of residuals (details supplied in Results section). Data also deviated from normality based on a Shapiro-Wilk test so they were arc-sin square root transformed and retested for normality. Normality and variance were improved by transformation and visual inspection of the residuals and a large sample size allowed parametric testing. A two-factor Analysis of Variance (ANOVA) was performed in R statistical analysis software using car and gplots packages to compare the proportion of bees present at each patterned feeder. Pattern and day were used as independent factors (R Development Core Team).

Experiment 2: Pattern differentiation and learning in a Y-maze

Forager honeybees were individually caught on Arizona State University Tempe campus. Foragers were identified by the presence of corbicular pollen sacs or were observed from a nearby nectar source and followed back to the entrance of the hive where a wire mesh barrier was set up to prevent their entrance to the hive box. They were captured individually and held in vials for up to one hour without food, but with access to a water-soaked cotton ball, and assigned a number. Then bees were given 10µl of a 50% sucrose solution per hour of waiting time. This amount was chosen to keep bees nourished, but not full, so that they would be willing to seek food rewards in the maze.

Then bees were randomly selected for maze-learning manipulations using a random number generator. The maximum wait time before testing was three hours. Bees were kept under fluorescent lighting near a brightly lit window at 25°C until beginning the experiment.

Maze Acclimation: Individual bees were initially placed in the maze for acclimation and allowed to explore until finding a reward in either patterned arm. Small white vial caps with 10 µl of unscented 50% sucrose reward were placed in the ends of each arm near the pattern to encourage bees to explore the full arm and pattern before finding rewards. Patterns and rewards were placed in the maze prior to bees. After finding the reward and tasting it (proboscis out and touching the reward cap), the bee was promptly removed before they could drink the full reward to ensure they remained motivated by hunger. The maze was cleaned after each trial with ethanol and allowed to dry to eliminate odor cues from bees walking or sucrose smearing on the maze. Each bee was introduced to the maze and allowed to explore ten times with the same pattern pair (location switched randomly for each trial using a random binary choice generator) to ensure acclimation and attention to the task. During the acclimation period, both patterns were associated with sucrose rewards. The pattern pair used to acclimate each bee was randomly chosen with a random number generator set (9 patterns available for 36 possible combinations). Bees were always acclimated to the maze using a different pattern combination than the pattern pair they were tested on later.

When first placed in the maze, bees flew erratically. Attention was not on task and bees were likely searching for escape routes as they were confronted with a novel

situation and enclosed in a non-social context (Matthews & Matthews 2010, Galizia et al. 2012). Because of this behavior bees were introduced to the maze ten times prior to testing in order to familiarize them to the maze and ensure that they were aware of food resources and the patterns in the maze. During each introduction, bees were allowed to consume 50% sucrose solution rewards associated with either pattern and both were equally rewarded. After the first few introductions, bees were relatively calm and tended to walk and explore the patterns more than fly around. Any bees that tended to choose a certain side (8/10 times or more) regardless of the position of the reward were excluded from the experiment due to site-fidelity. Any bees that did not calmly walk through the maze, and instead continued to fly erratically, persist in examining corners, or did not find the rewards during the acclimation period were also excluded. Once acclimated to the maze, bees were housed individually in a vial without food for 30-60 min to ensure they were hungry enough for the maze pattern differentiation task.

Pattern differentiation testing: Each bee was tested using only one pattern pair and the locations of each were switched randomly between trials using a random choice generator. Only one pattern was rewarded during the learning task. For half of the bees, the higher spatial frequency pattern was rewarded, while the other half were rewarded when they chose the lower spatial frequency pattern. A 10 µl water control was placed at the unrewarded pattern using the same type of vial cap apparatus so there would be no differing visual cues other than the patterns. Reward and control solutions were placed using different pipettes to avoid contamination. Rewards and controls were placed before bees entered the maze and were removed after every trip. The maze and reward/control

caps were cleaned with ethanol and allowed to dry between trips to prevent learning or avoidance based on odors (Zhang et al. 1996).

Each bee went through this task 10 times (trials) with each trial ending when the bee found either the reward or the control cap and sampled the reward sucrose or control water. Bees were promptly removed so the maze could be cleaned and patterns were switched randomly between each trip. Bees were forced to make a choice at a distance of 30 cm (the point at which the arms split and the bee had to travel down one arm or the other) from patterns. Once bees passed more than 5 cm into an arm, a choice was noted and the bee was allowed to proceed to the reward or control cap. Controlling choice distance was important for making predictions about differentiation and learning based on distance and visual acuity. If bees travelled through an arm but never went to the reward or control cap and turned around, they were removed to ensure they were not choosing that arm for any reason other than to select a pattern and visit the associated cap. A total of 20 bees was used for each pattern comparison (ten while rewarding the higher spatial frequency pattern and ten rewarding the lower frequency pattern) for a total of 200 bees. The average score (number of correct pattern choices) was calculated for each pattern comparison. Data were normally distributed variances did not differ significantly among the treatment groups ($p>0.05$). These data were compared using ANOVA with post-hoc Tukey's multiple comparisons tests using R (R Development Core Team 2012).

Assessment of pattern differentiation and learning abilities: In order to determine whether bees could differentiate patterns a logistic regression analysis and ANOVA of

the mean proportion of correct choices was performed for every pattern pair. When the mean of the number of correct choices was higher than 50% (≤ 50% represented random choice between the two patterns), bees could distinguish between the patterns in a pair. Logistic regressions on plots of choice (correct or not) vs. trial number were used to evaluate whether the bee tended to be more likely to choose the correct answer as the number of trials progressed. (Table 3; Wharton & Hui 2011). Learning abilities were also examined with logistic regression analysis using bee, trial, and pattern pair as factors. The initial analysis revealed that trial and pattern pair were significant. A subsequent logistic regression was performed using only the significant factors to assess interactions. Cumulative correct choice curves were also plotted vs. trial number as an additional index of learning, and the slopes of these lines compared among patterns as an index of the difficulty of the discrimination (Fig. 5). A mean slope close to zero among bees tested for a given pattern comparison indicated no learning whereas a slope above zero indicated some degrees of learning. Higher slopes indicate a faster learning rate than lower slopes.

Y-maze construction: A Y-maze was constructed of transparent acrylic sheeting. Acrylic panels used for the sides of the maze were 15.4 cm x 30.8 cm; these were bonded together using chloroform. At the end of each arm, I placed 15.4 cm x 15.4 cm acrylic panels with patterned discs. The maze was lined with Fluon™ to prevent bees from climbing on the glass. White Whatman™ filter paper was also placed along the outside walls of the maze so that bees could not look outside at other stimuli. Fluorescent lighting was overhead and the maze was placed near a large window to gain natural light.

Individually caught bees entered the Y-maze from the third arm which was closed with a panel after the bee entered the maze. When bees approached the Y-junction, both patterns were visible at the same time for visual assessment (Figure 2).

Visual pattern characteristics: The patterns used were 12.7 cm diameter discs with black and white radial lines that varied in spatial frequency. This style of patterns was chosen for ecological relevance to floral patterns, which are often radial. To avoid sensory bias for UV patterns (preference for specific features) toward previously learned or preferred floral colors, all patterns were laser printed on Whatman™ filter paper, which has low and consistent UV reflectance. Black and white were used because it is unlikely that bees had any previous experience with this color combination, and therefore, no preference for it. Entirely black, white, and gray discs were printed to determine whether bees preferred a certain intensity (ie. white is brightest and most intense and black is least bright and intense). Fully black and fully white patterns also have maximal contrast next to each other, eliminating contrast as an issue in identifying different patterns. The gray disc is most similar to the highest spatial frequency radial patterns, which may blend to a blurry gray based on the visual acuity and distance of bees from the pattern (Fig. 1, Table 1).

Black and white line size was kept the same within each pattern because it was unknown whether bees would pay attention to the black or white component. The spatial frequency of lines, or the number line cycles per wavelength for each pattern was calculated. The ratio of black to white area was measured using ImageJ (Table 1). The percent area of each pattern covered by black and white was determined by counting

pixel areas in ImageJ and used to choose a gray which would reflect the median intensity between black and white (Rasband 1997). Black, white, and an array of grays were printed on filter paper to measure reflectance using a coincident spectrophotometer (Johnsen 2016). The intensity of all gray shades were plotted from 250-750nm to determine the intensity of reflectance at UV and all visible wavelengths for bee vision. The weighted average of percent area covered by black versus white on all radial patterns was matched as closely as possible to a shade of gray with similar average reflectance. The shade of gray chosen resembles the supposed visual blurring of black and white at high spatial frequency as closely as possible. It is unknown whether the lines perceptually blur to black or gray for bees, so spectral measurements of the black and white areas of discs were used to calculate the spectral reflectance of gray which is the average of the reflectances of white and black (Fig. 1, Table 1). This gray was used to make a control disc to determine whether bees can perceive the difference in intensity and contrast among black, gray, and high frequency discs (Zhang et al. 1996, Land & Nilsson 2012).

Spatial line frequencies were chosen for patterns based on the calculated spatial resolution of honeybee compound eyes using 2.6º as the frontal interommatidial angle (Seidl & Kaiser 1981). The line width was equal to ½ λ, or ½ of the total wavelength. Then spatial frequency was calculated as 1/λ for each pattern based on the sizes of lines and linear distance between each line. Patterns above, below, and on the border of resolution for a given distance were chosen to create a continuum of visual similarity and differentiation difficulty (Table 2). Line components of patterns with high spatial frequency should blend together and be indistinguishable from the correct patterns,

thereby making differentiation between them much more difficult. The patterns which bees would be predicted to be able to distinguish were calculated using:

$$d\frac{\Delta\Phi}{360^{\circ}} = x\pi$$

Where $\Delta\ \phi$ is the interommatidial angle ($\Delta\phi$=2.6º), d is the distance the bee is from the pattern during inspection, 360° represents the complete visual field, and x is the distinguishable distance between lines that allows pattern differentiation, or the just noticeable difference in spatial resolution in degrees that represent the proportion of the visual field that needs to be occupied by an object in order to be resolved (Land 2011, Land & Nilsson 2012). To determine whether lines on a pattern with a given spatial frequency can be resolved, the lines are used to calculate the proportion of the visual field they occupy and compared with x. If smaller in width than x, lines on patterns should be blurry at the distance where the bee evaluates it (Table 2, [Land 2011, Land & Nilsson 2012]). If the proportion of the visual field occupied by one line in the pattern is larger than x, then bees should be able to see the distinct lines of the pattern.

Based on equation 1 and the spatial frequencies of the patterns, bees honeybees should be able to distinguish differences between the following pattern pairs: AI, BG, CD, CG, CI, and HI (Table 2). In these pattern pairings, the line spatial frequencies fall above the least noticeable difference threshold in honeybee vision. However, bees should not be able to differentiate between: DG, FG, GH, or II (control pairing). These pattern pairs, ranked by differentiation in spatial frequencies (and therefore, learning) difficulty from most to least difficult are: II, GH, DG, GC, CD, FG, HI, BG, AI, and CI (Table 2).

RESULTS

During the visual acuity test trials, bees would typically arrive at the Y-junction of the maze where both patterns were visible, walk in circles and look in either direction. When they entered an arm, bees typically stood in front of the pattern and walked onto it before going to the reward cap. When presented with very similar patterns, bees tended to fly more than bees presented with dissimilar or more easily distinguishable patterns (personal observations).

Bees were able to differentiate across the pattern pairs that were expected based on their visual acuity (Fig. 4), but to different degrees of accuracy. A Shapiro-Wilk test combined with a visual inspection of residuals indicated that data were fairly normally-distributed ($W=0.9439$, $p=0.0193$). Levene's test showed that data had equal variance ($F=0.3401$, $p=0.9558$, $df=9$). The mean score of all 20 bees from 10 trials per bee in each comparison ($N=200$) was compared across all ten pattern comparisons using ANOVA (Fig. 4) after examination of residuals for normality and equal variance. ANOVA demonstrated that the differences in mean score across comparisons were significant ($p<0.00001$, $F=126.13$, $df=9$). Logistic regressions results showed that patterns CI, HI, CG, and AI had intercepts significantly different from zero, showing that a higher number of correct choices for these pattern pairs.

It is unclear whether bees learned pattern associations. Logistic regression (Table 3) indicated that both trial ($p= 530e^{-7}$) and pattern pair ($p=8.62e^{-7}$) were significant factors in the regressions. Pair difficulty followed but was not significant ($p=0.08$ (Table 3]). Akaike's information criterion (AIC= 605.65, McFadden $R^2= 0.106$) indicates low

fit of the logistic model with four Fisher scoring iterations based on each factor included in analysis (trial, pattern pair, pair difficulty, and bee). Slopes of choice vs. trial were not different from zero ($p>0.05$) for any pattern pairs. However, very fast learning (within the first few trials) may have occurred for some pattern pairs; in this case, it is difficult to distinguish learning from differentiation because the slope would not be significant.

Free-flying honeybees had no preference for any pattern when all were presented with equal volume and quality rewards. Recruitment to patterns began within five minutes on each day and the first pattern bees landed on was not the same on any two days. These data were analyzed using two-factor ANOVA following an assessment of normality and variance. A Shapiro Wilk test and visual inspection of data residuals indicated that data were not normal ($W=0.89$, $p<0.001$). Results from a Levene's test for equal variance indicated that data were skewed ($F=1.84$, $p=0.0123$, $df=23$) so data were arc-sine square root transformed as proportions. Data transformation greatly improved the normality ($W=0.9770$, $p=0.0001$) and variance ($F=2.30$, $p<0.001$ $df=23$) of the data. Despite the remaining significance of the Shapiro-Wilk and Levene's tests after transformation, visual inspection of the residuals demonstrated that proceeding with parametric testing was appropriate due to the robustness of ANOVA with large sample sizes.

Two factor ANOVA revealed no significant differences in the proportion of bees visiting any pattern (Fig. 5; $p=0.1990$, $F=1.42$, $df=7$), though the effect of day was significant ($p=0.0079$, $F=4.93$, $df=2$). The interaction between the two factors was not significant ($p=0.3877$, $F=1.07$, $df=14$) and importance of day was driven by a larger

proportion of bees visiting white and lower frequency patterns than high frequency patterns, gray, and black on days 2 and 3 (Figure 3, Table 3). Since the interaction between day and pattern was not significant, all days were combined and pattern was compared again using ANOVA. Again, there were no significant differences among patterns ($p=0.05618$, $F=1.9943$, $df=7$; Figure 4, Tables 4 and 5), demonstrating that overall, bees did not prefer any of the patterns used.

DISCUSSION

Honeybee foragers were able to differentiate patterns in a manner consistent with their eye morphology. The results of the Y-maze behavioral tests generally matched theoretical predictions, with the percent of correct choices being higher for patterns predicted from eye morphology to be easier (Fig 4). Pattern pairs II, GH, CD, and DG were expected to be the most difficult to distinguish, and bees were not able to correctly choose the rewarded pattern more than 50% of the time in ten trials (Fig 4). Pattern pairs DG, CG, FH, and HI represented a medium level of theoretical difficulty, and for these pattern pairs, bees chose the reward-associated pattern 55-70% of the time. Pattern pairs CI, BG, HI, and AI should be the easiest to distinguish in theory, and for these pattern pairs, bees chose the reward-associated pattern 70-90% of the time. Pattern pair HI was fully gray vs. fully black panels (both with spatial frequency of one), and thus was likely distinguishable based on intensity rather than spatial frequency. There was a fairly continuous increase in the mean scores in order of pattern pair distinction difficulty (Fig 4). Exceptions are HI (discussed above) and BG in which bees sometimes flew directly

into the black area (pattern B). Despite this, it was clear that bees were able to distinguish well between the two patterns, as predicted. There were no pattern pairs in which bees were 100% accurate in choosing the correct pattern, even for the easier pattern pairings. This may be due to exploration testing whether or not bees could gain rewards in multiple locations in the maze.

It was unclear whether bees were able to learn to associate rewards with specific patterns over the number of trials allowed as shown by logistic regressions (Table 3) and cumulative choice curves (Fig 5). The number of trials may not have been sufficient to record learning, and in cases where patterns were very easily distinguished from each other, the task may have been too simple. There was a high proportion of correct choices but low logistic regression slopes. Trial and pattern pair were both significant factors in the logistic regressions. Further, Fig. 5 shows an increase in the slopes of learning curves with higher slopes for pattern pairs that were more easily distinguishable. The increase in slope was fairly continuous (slope= 0.04, Pearson's r^2= 0.691). Trial was important because, bees were more likely to choose correctly later than earlier. Pair difficulty was not a significant factor (p= 0.082, Table 3) but it is based on the difference in spatial frequency between pattern pairs. There were no differences among individual bees because bees demonstrating preferences for sides of the maze or specific patterns were removed during the acclimation period.

It is clear that honeybee behavioral differentiation abilities match their eye morphology in a continuous manner with more difficult comparisons resulting in fewer correct choices. There is no visual threshold, above which all distinctions are easy to

make and below which all comparisons are blurry and indistinguishable. This is likely due to the structure of the eye (Seidl & Kaiser 1981, Girufa & Vorobyez 1998, Land 2012). Images must take up a certain portion of the visual field in order to be distinguished as distinct items. At a distance of 30 cm where the choice was made, patterns with high spatial frequencies would have been blurry. The lines on patterns of similar spatial frequency would not have been noticeably different as bees assessed both patterns before making a choice (Land & Nilsson 2012).

These findings illustrate how honeybees may approach pattern comparison problems while foraging (Giurfa & Menzel 1997, Srinivasan 2010, Menzel 2012).

This study was done using bees walking in a maze, but pattern differentiation and learning becomes an even more difficult task while flying. However, wild bees are able to inspect objects at any distance in nature and are not restricted to inspect flowers from a distance greater than they can resolve. On the other hand, close visual inspection of all patterned targets is energetically wasteful and time-consuming (Schubert et al. 2002). Bees might neglect close visual inspection to avoid predation, aggressive interactions with conspecifics or heterospecific competitors, or to save energy (Avargues-Weber et al. 2011)). Additionally, the consequences of neglecting close visual inspection are few when resources are plentiful. In good circumstances, bees may choose not to closely inspect objects because alternative sufficient resources are nearby and the energetic cost to switch to that area is low. However, in circumstances with fewer resources, many predators, or a large number of competitors, visual inspection and the ability to distinguish patterns while flying is necessary (Greggers at al. 1997, Land 1997). The

results of this study show that eye morphology is a good predictor of visual acuity, and that the frequency of spatial patterns affects the differentiation abilities of honey bees in a relatively continuous manner.

TABLES & FIGURES

Table 1. Spatial properties of patterns used. Patterns 1-8 were used in both experiments but pattern 9 was only used in experiment 2 to add an additional low frequency pattern. Line width measurements were taken on 12.7cm diameter printed patterns. Wavelength is twice the line width and spatial frequency was calculated as the number of line cycles per degree (calculated as $1/TAN^{-1}$(wavelength/distance from pattern)). The percent of pattern area covered in black was measured in ImageJ using binary thresholding and pixel counts.

pattern label	pattern	line width (mm)	wavelength (mm)	spatial frequency (cycles/degree)	% area black
A		0.00	0.00	0	0.00
B		63.5	127.0	2.53	49.73
C		8.00	16.00	19.10	51.40
D		6.50	13.00	23.48	50.73
E		3.00	6.00	50.84	53.05
F		2.00	4.00	76.25	55.35
G		1.00	2.00	152.50	52.12
H		1.00	1.00	305.00	0.00
I		1.00	1.00	305.00	100.00

Table 2. Hypotheses for which pattern comparisons should be distinguishable at a distance of 30 cm based on the spatial frequencies of lines on each pattern, the difference between spatial frequencies in pattern comparisons (left lower half of table) and differences in brightness when there was no difference in spatial frequency between patterns (gray vs. black). Y (yes, green) and N (no, red) show which pattern comparisons should be distinguishable. Yellow indicates that pattern pair should be distinguishable based on brightness.

	A	B	C	D	E	F	G	H	I
A		Y	Y	Y	Y	Y	Y	Y	Y
B			Y	Y	Y	Y	Y	Y	Y
C				Y	Y	Y	Y	Y	Y
D			0.02		N	N	N	N	Y
E						N	N	N	Y
F							N	N	Y
G			0.44	0.42		0.25		N	Y
H							0.50		Y
I	1.0		0.94					0.00	N

Table 3. A) Logistic regression statistics for all pattern pairs showing that bees were unable to learn to associate rewards with patterns with the number of trials allowed. Positive slopes significantly different from zero would indicate learning. B) Logistic regression ANOVA of the interactions model show that trial and pattern pair were both significant factors in determining whether bees chose the correct pattern in a given comparison.

A pattern pair	spatial freq. diff.	slope	p	std. err	z	intercept	p	std. error	z
II	0.00	0.015	0.929	0.173	0.089	-1.335	0.0470	0.672	-1.986
GH	0.50	-0.209	0.215	0.169	-1.240	0.080	0.8956	0.612	0.131
CD	0.44	-0.065	0.709	0.174	-0.374	0.046	0.9414	0.631	0.074
DG	0.42	-0.081	0.636	0.170	-0.474	-0.302	0.6248	0.617	-0.489
CG	0.02	0.276	0.162	0.197	1.398	-1.709	0.0204	0.737	-2.319
FG	0.25	-0.209	0.215	0.169	-1.240	0.241	0.6948	0.613	0.392
CI	0.94	-0.251	0.205	0.198	-1.267	2.221	0.0184	0.942	2.357
BG	0.40	0.485	0.059	0.258	1.883	-1.543	0.0650	0.836	-1.845
HI	0.00	0.369	0.093	0.219	1.682	-1.595	0.0391	0.773	-2.063
AI	1.00	0.180	0.188	0.137	1.316	0.609	0.4044	0.731	0.834

B ANOVA coeff.	dev.	resid. df	resid. dev.	p
null model	NA	499	648.68	NA
pair difficulty	3.023	498	645.65	0.082

trial	20.644	497	625.015	**530e-06***
bee	0.195	496	624.81	0.659
pattern pair	45.15	487	579.65	**8.621e-07***

Figure 1. Visual properties of black, white and gray shades used in patterns. A) Reflectance of white, a series of grays, and black on Whatman filter paper used to make patterns with low UV reflectance. The light blue line indicates white and the red line indicates the spectral properties of the shade of gray used for the control gray pattern. The other lines show other shades of gray tested in order to choose the shade closest to medium intensity B) Average reflectance of white, black, and each gray shade with the gray used for the control pattern indicated by a red asterisk (*).

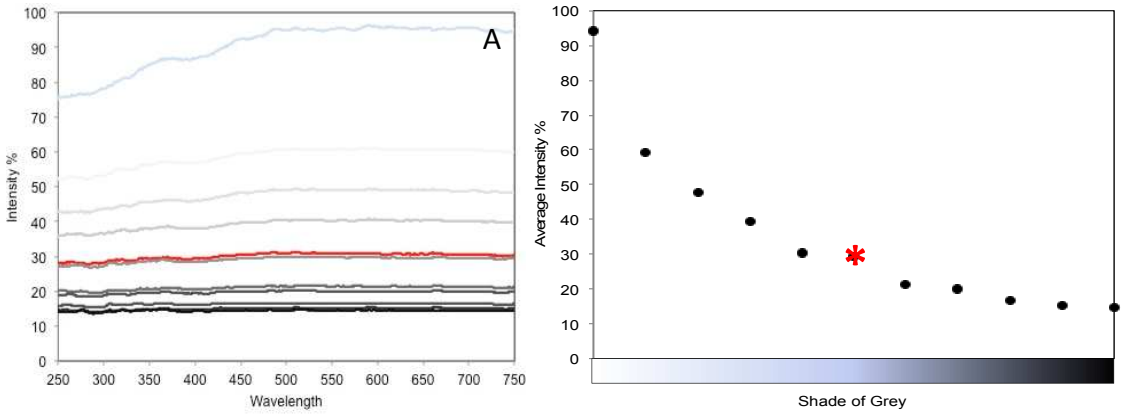


Figure 2. Schematic 3-D view of the experimental Y-maze with important areas highlighted. The black arrow indicates where the bee enters the maze. The dashed area represented where the honeybee must visually compare the patterns and decide which arm to explore. The small grey circle indicates where the reward or control water is associated with pattern. Patterns are shown on the vertical wall at the end of each arm.

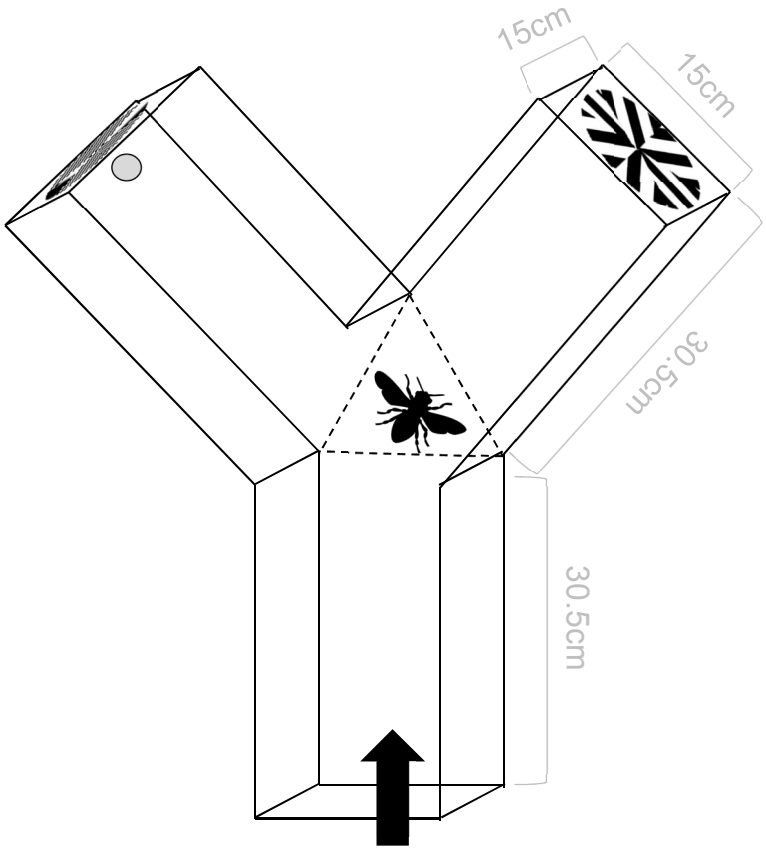


Figure 3. A) The proportion of free-flying bees visiting patterns with day1, day 2, and day 3 shown in green, blue, and red, respectively. Bees had no significant preference for any patterns (p=0.1990, F=1.4156, df=7), though the effect of day was significant (p=0.0079, F=4.9340, df=2) due to some degree of preference for white (pattern 1) when compared to black (pattern 8). Error bars represent ±standard error (SE=0.0040). Two-factor analysis of variance (ANOVA) showed that, while each day was significantly different, the proportion of bees at each pattern was not different on any given day and the interaction between day and pattern was not significant. This allowed elimination of day as a factor in analysis. B) The proportion of bees visiting patterns with all days combined. Bees had no significant preference for any patterns (p=0.05618, F=1.9943, df=7) though there is a trend indicating some degree of preference for white (pattern 1) when compared to black (pattern 8). Error bars represent ±standard error (SE=0.0040).

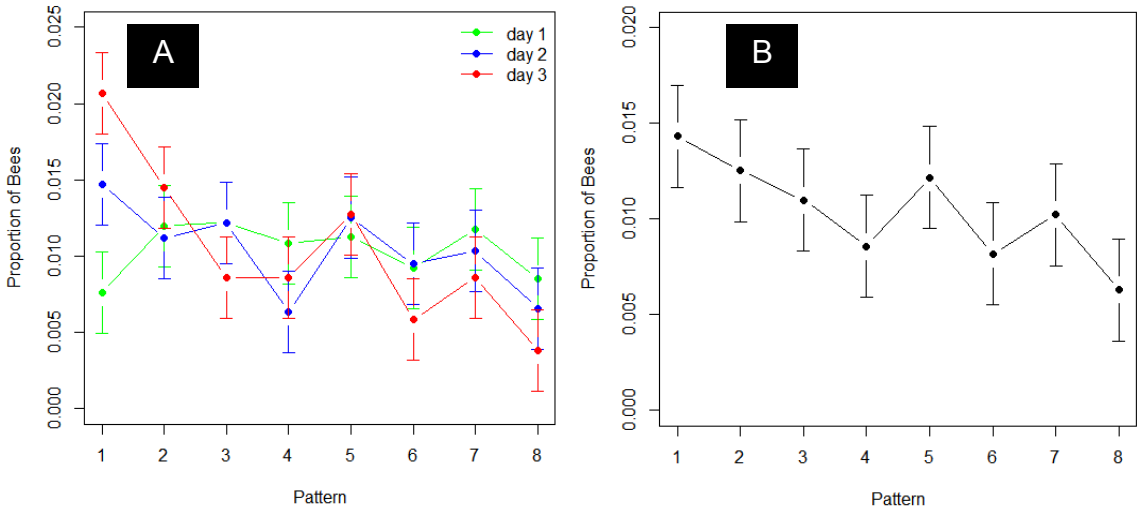


Figure 4. Differentiation abilities of honey bees increase continuously as pattern similarity decreases. Mean number of correct choices during ten trials for the ten bees tested with each pairwise comparison (n=20, total N=100). The patterns are arranged on the x axis according to theoretical difficulty in distinguishing patterns. Scores that are significantly higher than 5 (dashed horizontal line) indicate that bees were able to differentiate the patterns. Error bars represent ±standard error (SE=0.271). The letters a,b, and c show the statistical groups that scores fell within using ANOVA to compare among them.

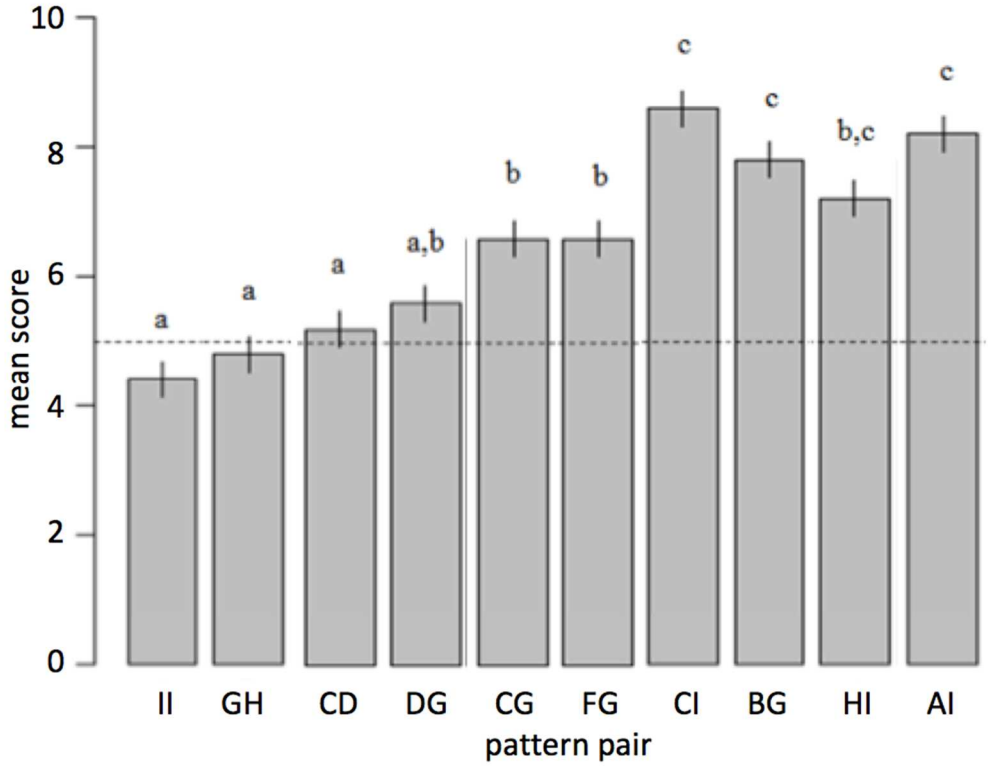
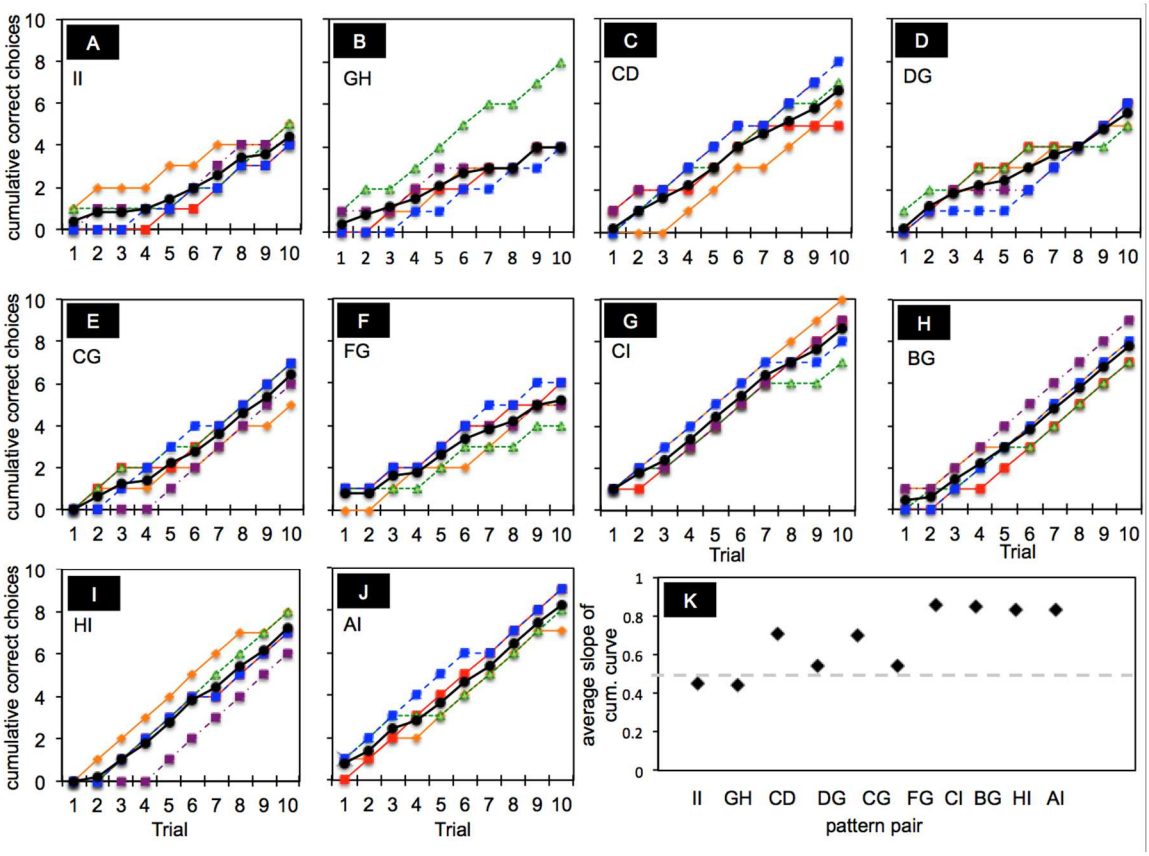


Figure 5. Cumulative learning curves for all pattern pairings in order (A-J) of difficulty. Pattern pairs are indicated below panel labels. For each, five bees (n=20/pattern pair) were chosen randomly from each group to demonstrate variability in the number of cumulative correct choices made. Each correct choice is an increase of one unit while incorrect choices were coded as zero. The average curve for each group is shown in each panel using black circles and lines. K) Average cumulative learning curve slopes plotted vs. the pattern pair arranged from left to right in order of theoretical difficulty in discrimination. Slopes close to the horizontal dotted line show low rates or learning and/or difficulty in distinguishing between patterns. Higher slopes show that bees chose the correct choice more quickly and reliably, resulting in slopes closer to one.



REFERENCES

Abramson, C. I., Cakmak, I., Duell, M. E., Bates-Albers, L. M., Zuniga, E. M., Pendegraft, L., Wells, H. 2013. Feature-positive and feature-negative learning in honey bees. *Journal exp. biol*, *216*(Pt 2), 224–229. doi:10.1242/jeb.069088

Avarguès-Weber, A., de Brito Sanchez, M. G., Giurfa, M., & Dyer, A. G. 2010. Aversive reinforcement improves visual discrimination learning in free-flying honeybees. *PloS one*, *5*(10), e15370: 1–11. doi:10.1371/journal.pone.0015370

Avargues-Weber A, Deisig N, Girufa M. 2011. Visual cognition in insects. *Annu. Rev. Entomol*. 56: 423-443

Avarguès-Weber, A., Mota, T., & Giurfa, M. (2012). New vistas on honey bee vision. *Apidologie*, *43*(3), 244–268. doi:10.1007/s13592-012-0124-2

Benard, J., & Giurfa, M. 2008. The cognitive implications of asymmetric color generalization in honeybees. *Animal cognition*, *11*(2), 283–93. doi:10.1007/s10071-007-0112-5

Galizia, C. G., Eisenhardt, D., Giurfa, M. 2012. *Honeybee Neurobiology and Behavior: A Tribute to Randolf Menzel*. New York: Springer Science & Business Media.

Giurfa, M., Hammer, M., Stach, S., Stollhoff, N., Müller-deisig, N., & Mizyrycki, C. 1999a. Pattern learning by honeybees: conditioning procedure and recognition strategy. *Animal behaviour*, *57*(2), 315–324. doi:10.1006/anbe.1998.0957

Giurfa, M., & Menzel, R. 1997. Insect visual perception: complex abilities of simple nervous systems. *Current opinion in neurobiology*, *7*(4), 505–13. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9287201

Giurfa, M., & Vorobyev, M. 1998. The angular range of achromatic target detection by honey bees. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology*, *183*(1), 101–110. doi:10.1007/s003590050238

Giurfa, M., Zaccardi, G., & Vorobyev, M. 1999b. How bees detect coloured targets using different regions of their compound eyes. *Journal of Comparative Physiology A: Sensory, Neural, and Behavioral Physiology*, *185*(6), 591–600. doi:10.1007/s003590050420

Greggers, U. W. E., Mauelshagen, J., & Berlin, F. U. 1997. Matching behavior of honeybees in a multiple-choice situation : The differential effect of environmental stimuli on the choice process Matching behavior of honeybees in a multiple-choice situation : The differential effect of environmental stimuli on the , *25*(4).

Hempel de Ibarra, N., Giurfa, M., & Vorobyev, M. 2002. Discrimination of coloured patterns by honeybees through chromatic and achromatic cues. *J. comp.physiol. A, Neuroethology, sensory, neural, and behavioral physiology*, *188*(7), 503–12. doi:10.1007/s00359-002-0322-x

Johnsen, S. 2016. How to measure color using spectrometers and calibrated photograph. *J. Exp. Biol.* 219: 772-778; doi: 10.1242/jeb.124008

Land, M. F. 1997. Visual Acuity in Insects. *Annu Rev Entomol*, *42*(46), 147–177.

Land, M.F., Nilsson, D. 2012. *Animal Eyes* (2nd ed.). Oxford: Oxford University Press.

Matthews, R.W., Matthews, J. R. 2010. *Insect Behavior* (2nd ed.). New York: Springer Science & Business Media.

Menzel, R. 2012. The honeybee as a model for understanding the basis of cognition. *Nature reviews. Neuroscience*, *13*(11), 758–68. doi:10.1038/nrn3357

Neal, P. R., Dafni, A., & Giurfa, M. 1998. Floral symmetry and its role in plant-pollinator systems: terminology, Distribution, and Hypotheses. *Annu. Rev. Ecol. Syst.*, *29*(1), 345–373. doi:10.1146/annurev.ecolsys.29.1.345

R Development Core Team. 2012. R: A Language and Environment for Statistical Computing, version 2.15.1. R Foundation for Statistical Computing, Vienna, Austria: http://www.R-project.org

Rasband, W.S. 1997-2012. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/

Schubert, M., Lachnit, H., Francucci, S., Giurfa, M. 2002. Nonelemental visual learning in honeybees. *Animal Behav*. 64(2): 175-184

Seidl, R., Kaiser, W. 1981. Visual field size, binocular domain and the ommatidial array of the compound eyes in worker honey bees. *J Comp Phys A*, 143: 17-26

Srinivasan, M. V. 2010. Honey bees as a model for vision, perception, and cognition. *Annu rev entomol*, *55*, 267–84. doi:10.1146/annurev.ento.010908.164537

Stach, S., & Giurfa, M. 2001. How honeybees generalize visual patterns to their mirror image and left–right transformation. *Animal Behaviour*, *62*(5), 981–991. doi:10.1006/anbe.2001.1839

Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S. B., & Menzel, R. 2001. Colour thresholds and receptor noise: behaviour and physiology compared. *Vision research*, *41*(5), 639–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11226508

Warton, D. I. and F. K. C. Hui 2011. "The arcsine is asinine: the analysis of proportions in ecology." *Ecology* 92(1) 3-10.

Zhang, S. W., Bartsch, K., & Srinivasan, M. V. 1996. Maze learning by honeybees. *Neurobiol learn and mem*, *66*(3), 267–82. doi:10.1006/nlme.1996.0069

CHAPTER 2

SIZE IS ONLY A NUMBER (SOMETIMES): SMALL STINGLESS BEES SPECIES EQUAL LARGER SPECIES IN VISUAL LEARNING TASKS

ABSTRACT

Body size miniaturization, an evolutionary phenomenon that occurs when species become smaller than ancestors over time, likely imposes behavioral constraints and challenges for animals. Miniaturized animals have smaller brains and sensory system structures such as eyes and antennae, and thus would be expected to lead to poorer behavioral performance. However, smaller animals have relatively larger brains and sensory structures, and the few prior studies that exist have found little evidence for an effect of size on behavioral capacities. It is challenging to assess the effect of body size on the sensory and brain performance of animals as it can be difficult to find related species that differ strongly in size yet retain similar life histories allowing standardized behavioral tests. Here we overcome these challenges using ten stingless bee species that varied from 1-115mg in body mass. Smaller species had smaller brains and eyes in an absolute sense, but those structures were relatively larger in the smaller species. Based on their smaller ommatidia and lower optimal interommatidial angles, smaller species should have poorer visual acuity. We used a Y-maze and achromatic visual patterns that varied in spatial frequency of pattern lines (see Chapter 2) to test the visual discrimination and associative learning capacities of each species. We found that smaller species performed similarly to larger species in both differentiation (ability to

differentiate between two patterns when one was associated with a reward) and learning tasks (the number of trials to reach an asymptote of correct choices between patterns). Thus, at least for these tasks, smaller stingless bee species achieved similar behavioral abilities despite their smaller brains and eyes. Understanding the mechanisms by which smaller animals compensate for their small neuro-sensory systems will have broad significance for understanding the evolution of body size and for development of simple yet efficient artificial intelligence systems.

INTRODUCTION

Blue whales are billions of times larger than fruit flies, illustrating the incredible variation in animal body size. Yet, these and all animals must perform the same general behaviors to survive: mating, locomotion, and foraging. For most of these behaviors, sensing the environment and learning are necessary to successfully complete these essential functions. Despite the critical importance of these behavioral capacities, we understand little about the scaling of sensory and learning performance across species that differ in size.

There is some behavioral evidence that larger animals with larger brains (absolute size) have greater learning capacities than smaller animals. Within species, there is a great deal of evidence suggesting that a bigger brain is needed for complex behaviors such as learning (Hahn 2006, Niven 2010, Niven & Farris 2012). Some studies indicate that larger brains allow animals a greater range of behavioral options and higher performance (Eberhard & Wcislo 2011, Hahn 2006, Kaas 2000), but this evidence typically uses pairs of unrelated species (rather than controlled phylogenetic studies) or examines other aspects of life history, such as niche specialties, that are not dependent on body size. Some studies have shown that larger animals make more accurate decisions in learning transference and reverse discrimination experiments (Riddell et al 1977, Gossette 1969). These studies have tentatively shown that the bigger brains of bigger animals (among species) are better for behavioral performance using primates (humans and other apes, squirrel monkeys), rodents (mice, rats and shrews, and fish [Isler 2013, Campi & Krubitzer 2010, Kortschall et al. 2013), and have led to the suggestion that

limits on behavioral capacities may constrain the lower size limit of brains and animals (Grebennikov 2008). However, none of these studies have examined the effects of body size across a phylogenetically controlled broad range of animals (instead using one or few species) or intentionally studied the effects of body size, although they did examine life history and ecological differences.

Morphological studies of the scaling of brains suggest that animals morphologically compensate, at least partially, for their absolutely smaller sizes. Across most taxa studied to date, smaller species have relatively larger brains, a trend known as Haller's Rule (Rensch 1948). In the most extremely miniaturized species, this trend can lead to brains larger than heads. Tiny orb-weaving spiders retain brains so large that they extend into the thorax and legs (Eberhard 2007, 2011). Plausibly, natural selection drives this trend, with smaller species being under greater selection for larger brain sizes due to possible behavioral limitations imposed by small brains (Hanken & Wake 1993). Conceivably, the relatively greater brain size, and other types of compensatory changes (e.g. smaller neurons, more synapses, etc.) could allow smaller animals to achieve similar behavioral capacities as larger animals. However, there is mounting evidence that absolute brain size and brain size relative to body mass may not be good predictors of behavioral abilities after all. In salamanders are rodents, there is no evidence that the size of behavioral repertoires decreases in smaller brained animals (Campi & KrubitzerHanekn & Wake 1993, 2010, Roth et al. 1995, Eberhard 2011).

There is considerable evidence that "bigger is better" in the structure of sensory systems. In the olfactory systems of insects, body size correlates positively with antennal

sensitivity and how insects behave in response to odors (Spaethe & Brockman 2007). Larger eye size usually correlates with better vision (resolution and acuity) among animals of different sizes and different ecologies among animals of similar size (Peters 1983, Chapman 1982, Spaethe & Chittka 2003). Smaller animals typically have smaller (though proportionally larger) eyes with lower visual acuity (Dusenberry 1992, Kiltie 2000). Bigger eyes are associated with greater visual sensitivity, acuity, and related behaviors (Peters 1983, Chapman 1982, Spaethe & Chittka 2003). In insect apposition compound eyes, larger eyes have a greater number of ommatidia (facets), and thus a larger density of photoreceptors. These ommatidia may also be larger, allowing greater light sensitivity (Jander & Jander 2002). Interommatidial angles (the angle between adjacent ommatidia) dictate the resolution of images. The finest image grating an animal can resolve is approximately twice the interommatidial angle (Land 1989). As eyes get smaller, facet size must also get smaller to accommodate the smaller interommatidial angles that are better for resolution, but this results in lower sensitivity to light (Land & Nilsson 2002). Given these body size relationships, smaller animals might be less behaviorally capable due to poorer visual resolution or sensitivity.

Despite evidence suggesting that small and miniaturized animals are often limited behaviorally by their size, there are indications to the contrary. Most animals on earth are small (Blackburn & Gaston 1994) and miniaturization, the evolution of smaller body size relative to ancestral species, occurs in every animal clade (Hanken & Wake 1993). Small animals like bees, ants, and other invertebrates are excellent models for studying social complexity and learning (Menzel 2013, Eberhard & Wcislo 2011). Humans weigh much

less, on average, than gorillas, and often less than orangutans but have more complex tool use, social complexity, and learning behaviors (Herculano Houzel, 2009, Gibson et al. 2001). Whether are not small animals are truly limited behaviorally, in any type of behavior, is unresolved. Learning studies have historically been especially difficult to do across animal species differing substantially in body size, as these have often failed to compare analogous behaviors or conflated life history differences with differences in learning abilities (Gossette 1969, Harvey et al. 1980, Kortschall et al. 2013, Herculano-Houzel 2009, Campi & Krubitzer 2010). To date, no studies have determined the scaling of learning behaviors across species in a controlled manner. A primary reason for this lack of knowledge is that it is difficult to find clades of animals with a wide size range that do not differ strongly in ecological niche or life history (Peters 183, Hanken & Wake 1993, Eberhard & Wcislo 2011, Bonner 1979).

In this study, I document the scaling of visual learning abilities among ten stingless bee species that vary in body size from 1-115mg in mass, including species that have undergone miniaturization from larger ancestor species. The species chosen have similar life histories and ecological roles as highly social, generalist pollinators, similar to honeybees. A fairly well resolved phylogeny for the stingless bee clade (Meliponini) allowed phylogenetically controlled comparisons (Rasmussen & Cameron 2012). I measured head and brain mass, and also eye morphology to establish the theoretical scaling of visual acuity. We find that, confounding expectations, smaller bees have similar visual discrimination and visual associative learning capabilities as larger bees,

which could be partially explained by relatively greater investment in brain and sensory structures.

METHODS

Stingless bee foragers were identified and caught while foraging at flowers or honey water feeders, or returning to nest locations in Gamboa, Panama City, Barro Colorado Island, and Santa Rita Arriba in the Republic of Panama. Bees were kept in single species groups of five or fewer individuals in 50 ml centrifuge tubes with access to sugar water until they could be transported to back to Smithsonian Tropical Research lab facilities in Gamboa, Panama. Species collected, from smallest to largest, included *Plebeia franki, Plebeia frontalis, Tetragonisca angustula, Frieseomelitta nigra, Scaptotrigona panamensis, Scaptotrigona lutepeinis, Trigona muzoensis, Trigona fulviventris, Melipona panamica, and Melipona triplaridis* (Fig. 1). Then bees were separated, numbered, and kept individually in smaller centrifuge tubes with a 50% sucrose solution in a cotton ball. One hour before experimentation, food sources were replaced with water to encourage searching for food rewards in the maze. All bees were used for maze experimentation on the day they were caught.

Bees of each species were selected randomly for experimentation using a random number generator that chose among the available bees daily. If selected, each bee was placed in the maze for acclimation. During this acclimation period, each side of the Y-maze (Fig. 2) contained a visual pattern and a 50% sucrose reward placed on a small glass vial cap. Bees were allowed to search the maze freely until they found a reward and then were removed from the maze. The maze, pattern, and reward cap were cleaned with

ethanol, allowed to dry, and replaced in the maze before the bee was reintroduced. The positions of each visual pattern were switched between introductions. Each bee was introduced to the maze in this manner 5-20 times. If a bee displayed side or pattern-preferences, it was removed from the experiment and not used afterward (see Chapter 2).

Pattern distinction test: Following acclimation, individual bees were tested on which patterns they could distinguish from each other. To do this, each bee was randomly assigned a pattern pair (Table 2), which may or may not have been the pair they were acclimated to. Each bee individually entered the maze (Fig. 2) and was allowed to visually assess the patterns. Both patterns were visible from a distance of 30-35 cm away in the Y-junction of the maze. Once a bee entered an arm of the maze (a distance of 30 cm from the pattern), a pattern choice was recorded as correct (rewarded with 50% sucrose) or incorrect (not rewarded but with water available). After a choice was recorded, each bee was allowed to continue through the maze until they found the reward or water cap in the arm they had chosen. For larger species, the volumes for water and 50% sucrose were 10 µl (*M. panamica* and *M. triplaridis*), medium bees were given 5 µl (*T. fulviventris, T muzoensis*, *S. panamensis, S luteipenis,* and *F. nigra*), and the smallest species were given 2.5 µl (*T. angustula, P. franki*, and *P. frontalis*). These volumes were chosen to be substantial for reinforcement but not such that would leave the bee unwilling to continue searching for food. Twenty bees/species each experienced ten trials. Half of the 20 bees of each species were tested while rewarding one pattern and half were tested while rewarding the other pattern in the pair to control for any intrinsic pattern preferences.

Pattern learning test: In order to determine if and how quickly bees were able to learn the association between specific patterns and sucrose rewards, individual fresh bees were allowed to complete as many trials through the maze as necessary until they successfully chose the rewarded pattern ten trials in a row. The same pattern pairs were used as in the pattern distinction test (Table 2). The same criteria were also used to record bee's pattern choices. Bees were acclimated to the maze as described above for the pattern distinction test. The pattern pair presented to any given bee in the learning test was not the same pattern they were acclimated to, but five bees for each species were used.

Maze construction: The maze used was a modified Y-maze made of transparent acrylic panels. Acrylic panels used for the sides of the maze were 15.4 cm x 30.8 cm; these were bonded together using chloroform, as in (Duell et al.; Chapter 2 of this dissertation). Only two arms were constructed. A third entry arm was not used because I found that stingless bees spent a lot of time exploring it rather than at the task of choosing an experimental arm. At the end of each arm I placed 15.4 cm x 15.4 cm acrylic panels with black and white patterned discs (Fig. 2). The maze was lined with Fluon to prevent bees from climbing on the glass. White Whatman™ filter paper was placed along the outside walls of the maze so that bees could not look outside at other visual stimuli. Fluorescent lighting was overhead and the maze was placed near a large window to gain natural daylight throughout the task with each arm gaining equal access to daylight (Duell et al; Chapter 2 of this dissertation).

Patterns: Black and white patterns were modified from those used in Chapter 2 and consisted of black and white parallel line patterns printed with a laser printer on white, low UV Whatman™ filter paper (Table 1). Parallel lines were used instead of radial lines because stingless bees tended to fly directly at the black portions of the radial line patterns. The lines on each pattern differed in spatial frequency. In this case, bees of different species were expected to differentiate and learn different pattern pairs based on their visual systems. Completely white, black, and gray discs without lines were also made. The pair of gray vs. gray was used as a negative control that no bees should have been able to differentiate while white vs. black was used as a positive control that all bees would have been able to differentiate. The level of difficulty of each pattern pair (which patterns each species should and should not be able to differentiate based on their eye morphology) was determined by calculating the difference in spatial frequency between patterns and subtracting it from the visual acuity of each species (Table 1, 2).

Visual morphology: To assess visual acuity, eye morphology was measured on pinned stingless bee forager specimens retained after the maze experiment using three to five bees/species for all ten species using a Canon EOS 7D Mark II digital SLR camera with 65mm Canon macro lens. Each pinned bee was mounted individually on white or black modeling clay with white or black paper as a background for the photos, depending on which color most contrasted with eye color. Most species had black eyes, so white paper was used, but some (*T. muzoensis*, *P. franki*, and *T. angustula)* have light yellow green to orange eyes. The camera was connected to the Zerene Stacker program so that images could be taken remotely. All vibrations were kept to a minimum and the only

light source was camera flash. A stack of 50-100 photos of the left eye of each bee was taken from frontal, lateral, and dorsal perspectives for each bee. The digital magnification multiplier, F-stop, step size, dwell time, and number of steps (photos) were determined individually for each bee. The image stack was rendered and compressed for maximal contrast and focus on ommatidia. In ImageJ, the number of ommatidia was measured by placing colored dots on the center of each ommatidium and counting them all. Ommatidial widths and angles were measured on the frontal region of the right eye for each bee, using a method adapted from Bergman & Rutowksi (2016). To measure ommatidium width, a separate photo was taken for each bee at the same magnification. A line was drawn across five ommatidia and measured in pixels, then divided by five to get the average size of a single ommatidium. This was done five times for each bee. Interommatidal angles were measured by placing the angle tool in ImageJ through the center of an ommatidum on the edge of the eye in dorsal view (that showed angles for the frontal ommatidia of the eye). The other line of the angle tool was placed on five ommatidia away. Then the angle between the two lines was measured and divided by five. This was done for five angles per bee. As these methods were developed for insects with rounder eyes (butterflies), we first performed these measurements using honeybees. The measurements recorded for ommatidial width and angle matched published values (Jander & Jander 2002, Land & Nilsson 2002), confirming the utility of this approach for bees.

The patterns that each species should be able to resolve was calculated for each species using equations from Land & Nilsson 2012 and Land 1989 that incorporate the

interommatidial angle, distance from the patterns during evaluation, and spatial frequency of lines on the patterns (see Chapter 1 methods for additional details). Hypotheses for which patterns bees of different species should have been able to differentiate are found in Table 2. Visual acuity was also assessed with PGLS to determine the relationship between body size and acuity measures while assessing phylogenetic signal, and whether visual acuity played a role in determining which patterns were differentiated and learned by different species (Table 3).

Additional morphology measures: A subset of 10 bees/species was retained for additional measurements that included wet body mass, head mass, and brain mass. These data were used to verify whether stingless bees follow Haller's rule in which smaller organisms have larger heads and brains proportional to their body mass, and whether head and brain mass contribute to differences among species in learning and differentiation abilities. The relationship of brain and head masses to body mass were assessed by PGLS (Table 3).

Statistical analysis: Scaling data for pattern differentiation and learning among species, in order of body mass, were analyzed first with logistical regression because data were proportional. Since there is currently no accurate way to assess proportional categorical data while controlling for phylogenetic signal, the mean values for each species (in order of average mass) was assessed using phylogenetic generalized least squares regression (PGLS) to account for phylogenetic signal for the proportion of correct choices in differentiation and learning trials, and differences in proportion of correct choices among all pattern pairs vs. average body mass for each species (Revel

2010 [Table 3]). Cumulative choice curves for all species were constructed for the learning test (Fig.4) and the mean number of trials to proficiency was calculated or each species. These data were then plotted vs. mean body mass and assessed with PGLS (Table 3). PGLS was also performed to assess the scaling of head mass, brain mass, ommatidia width, ommatidia number, and interommatidial angles.

RESULTS

Pattern differentiation abilities did not vary across species or mass (Fig. 4,5, Table 3). Phylogenetic signal was low (not different from zero for all pattern comparisons using PGLS analysis across species) indicating that body mass and pattern differentiation abilities were not driven by phylogeny. The number of trials needed for bees to choose the correct pattern in a pair ten times in a row was significantly lower in smaller species [Table 3, Fig. 4, 5]), suggesting faster learning in smaller bees. Therefore, smaller species did not reach greater proficiency (proportion correct) in learning to associate a reward with a particular pattern but they did learn the pattern more quickly.

Smaller stingless bee species had relatively large heads (slope of log head mass on log body mass = 0.827, $p = <0.001$ compared to isometric slope = 1) and relatively larger brains (slope = 0.543, $p<0.001$ compare to slope = 1). Since the hypometric slope of brain mass scaling is lower than that of head mass scaling, brains took up relatively more space in the heads of smaller bees. Both slopes had no phylogenetic signal ($\lambda= 0$, $p(\lambda)= 1.00$ [Table 3]). Larger bees had more and wider facets (Table 3, Fig. 6), but facet width increased much more slowly with size than predicted by isometric scaling (slope of

log facet width on log body mass = 0.115). Again phylogenetic signal was low (not significantly different from zero, p=0.124 for ommatidial number and p=1.00 for ommatidial width (Table 3, Fig. 6)). Interommatidial angles were consistent across body mass variation (slope=0.022, p=0.597) and there was low phylogenetic signal that was not significantly different from zero (λ= 0.227, p(λ)= 0.761 [Table 3, Fig. 6]).

DISCUSSION

Small stingless bee species performed similarly in terms of pattern differentiation abilities and proficiency (proportion of correct choices). They learned faster than larger species (number of successive trials needed to achieve ten correct choices in a row). This indicates that learning abilities and associated behaviors are preserved despite the hypothetical limits of small size, and likely contribute to improving competitive abilities relative to larger species in a more natural context. I also show that smaller species have relatively larger heads and brains (hypometric scaling) and more and larger ommatidia than expected for their size. These may be structural compensations that allow for the preservation of learning abilities despite small size.

Similar performance in pattern differentiation and learning proficiency refutes claims that smaller stingless bees are cognitively or behaviorally limited by their size, at least in a visual learning context. This conclusion is strengthened by the lack of phylogenetic signal for body size and learning and differentiation variables. The ability of small bees to discriminate and learn visual patterns as well as closely-related, much larger species may be partly explained by their relatively larger brains and scaling of eye

morphology that preserves ommatidial angle and visual acuity across two orders of magnitude in body size.

I provide clear evidence for morphological adaptations that must aid preservation of these behavioral capacities across size. In smaller stingless bee species, brain tissue took up a greater proportion of head space as shown by the difference in hypometric scaling slopes, with brain mass being even larger in proportion to body mass and head mass in smaller species. Haller's rule was also shown in the relatively larger eyes of smaller species; this is consistent with trends found in other small animals across clades, such as salamanders in which the smallest species have the largest eyes relative to body size but the largest species have the next biggest eyes (Linke et al. 1986). Larger eyes and brain proportional to body size may be compensatory, as isometric scaling of these features might result in eyes with sensitivity too low for resolving patterns and neuropils without the necessary structural elements and size for processing the visual information needed to differentiate and learn patterns. The lack of scaling of interommatidial angles is not surprising given that interommatidial angles directly correlate with resolving power (Land & Nilsson 2012, Land 1989), though it has been proposed that visual acuity should be less in smaller species to hypothetically smaller eyes in terms of absolute size which should cause lower light sensitivity and, probably, resolution owing to hypothetical spatial constraints on the number and size of ommatidia (Eberhard & Wcislo 2011). However, Perl & Niven (2016) have found that different areas and characteristics of the insect eye can respond differently to body size. Therefore, optimization within a certain limit of interommatidial angles may help optimize resolution (Perl & Niven 2016, Land

& Nilsson 2012). Overcoming the problems of small body size in learning visual stimuli may require greater visual acuity in certain areas of the eye and varying the types of photoreceptors present in each area, as observed in Hymenoptera and Lepidoptera (Land & Nilsson 2002, Land 1989, Rutowski 2002). I solely measured the frontal region of the eye that would look directly at the patterns, but further work should be done to characterize the vision of stingless bees.

It is also possible that there are behavioral mechanisms that explain, or at least contribute to, the ability of small bees to do as well as large bees in pattern differentiation. These include, but are not limited to, taking a greater length of time to examine patterns before making a choice or using head movements to view patterns at various angles (Land & Nilsson 2012, Land & Tatler 2009, Rossel 1980). It may be that smaller stingless bee species needed fewer trials to learn pattern associations because they use a different decision-making process than larger species. This would be particularly advantageous in floral foraging situations where small bees may be kicked off flowers by larger bees through brute force. If smaller bees can make decisions faster, and stick with correct decisions more easily, they would be able to compete more effectively.

Further work should explore variation in the volumes and structure of neuropils within the brain that are responsible for learning, such as mushroom bodies and optic lobes in insects. Many argue that this is necessary because specific signals and behaviors are processed in certain areas of the brain. Among rodents and salamanders studied, overall brain size does not correlate with behavioral abilities (Campi & Krubitzer 2010,

Roth et al. 1995). Instead, only the brain areas related to important behavioral tasks correlate with abilities (Campi & Krubitzer 2010, Menzel 2013). Between nocturnal and diurnal rodents, for example, diurnal rodents have a larger sensory cortex associated with visually driven behaviors (Campi & Krubitzer 2010). Large mushroom bodies are associated with learning, memory, and social behaviors among hymenoptera (reviewed in *Invertebrate Learning & Memory* 2013), and a larger frontal lobe is associated with greater intelligence and larger behavioral repertoires in apes.[16] These correlations are due to mosaic evolution of the brain in which different areas evolve to different sizes based on selection on the abilities associated with those areas (Eberhard & Wcislo 2011, Barton & Harvey 2000). Some have found that areas of the brain associated with specific, important behaviors are relatively larger regardless of body size (Iwaniuk et al. 2000, Herculano-Houzel et al. 2006, Miklos 1998).

This study shows that small body size is not necessarily a limitation for cognitive tasks, an important finding in determining why small size among animals commonly evolved and persists. Maintenance of behavioral proficiency at small size improves competitive abilities. Future work should further examine the mechanistic underpinnings and additional compensations (physiological and behavioral) needed, if any, for maintaining complex learning abilities while small.

TABLES & FIGURES

Table 1. Parallel line patterns and their spatial characteristics

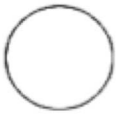
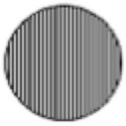
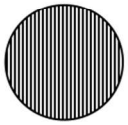
pattern label	pattern	line width (mm)	Wavelength (mm)	spatial frequency (cycles/min)
A		0.00	0.00	0.00
B		26	52	0.019
C		17	34	0.029
D		8.6	17.2	0.058
E		3.4	6.8	0.147
F		0.85	1.7	0.588
G		1.00	NA	NA
H		1.00	NA	NA

Table 2. Predictions for which patterns pairs should be visually differentiated, and therefore learned by different stingless bee species based on visual acuity (calculated using interommatidial angles), distance from patterns during visual evaluation (30cm), and spatial frequency of patterns.

pattern pair	spatial freq. diff.	species
AH	1.00	*M. triplaridis* *M. panamica* *T. muzoensis* *T. fulviventris* *T. angustula* *P. franki* *P. frontalis* *F. nigra* *S. panamensis* *S. luteipenis*
HF	0.41	*M. triplaridis* *M. panamica* *T. muzoensis* *T. fulviventris* *T. angustula* *P. franki* *P. frontalis* *F. nigra* *S. panamensis* *S. luteipenis*
BC	0.01	*M. triplaridis* *T. muzoensis* *T. fulviventris* *P. franki* *P. frontalis* *T. angustula* *F. nigra*
CD	0.03	*M. triplaridis* *P. franki* *P. Frontalis* *T. Angustula* *F. nigra*
FG	0.41	*M. triplaridis*
EF	0.44	*M. triplaridis*
FG	0.41	*M. triplaridis*
DE	0.09	none
GG	0.00	none

Table 3. Phylogenetic Generalized Least Squares Regression (PGLS) analysis of the body mass scaling of pattern differentiation, learning, and morphological scaling. All parameters were log-transformed and tested for linear relationships with log body mass, correcting for phylogeny. Whether tested across all patterns or any of the individual patterns, there was no effect of body size on the ability to discriminate visual patterns. Smaller stingless bees were significantly faster than larger species in achieving proficiency (ten correct choices in a row). The scaling of head mass and brain mass were hypometric, indicating that smaller stingless bees had proportionally larger heads and brains. Smaller species also had larger eyes (# facets and facet width) relative to body mass., but not in terms of absolute size. Lambda (λ) indicates the degree of phylogenetic signal and p(for λ = 0)) shows whether there is a significant phylogenetic signal.

parameter	slope	intercept	std. err.	t	p	λ	p (for λ = 0)
% correct	-0.012	-0.160	0.021	-0.587	0.576	0.296	0.696
mean # trials to proficiency	0.208	1.903	0.081	2.578	0.035*	0.374	0.803
pair 1 (AH)	-0.006	-0.339	0.022	-0.264	0.799	0.000	1.000
pair 2 (HF)	0.006	-0.310	0.025	0.236	0.820	0.000	1.000
pair 3 (BC)	-0.006	-0.346	0.022	-0.295	0.777	0.000	1.000
pair 4 (CD)	-0.034	-0.390	0.018	-1.866	0.104	0.000	1.000
pair 5 (DE)	-0.032	-10.384	0.028	-1.115	0.302	1.000	0.378
pair 6 (EF)	0.013	-0.290	0.028	0.475	0.649	0.000	1.000
pair 7(FG)	0.009	-0.296	0.028	0.301	0.773	0.000	1.000
pair 8 (GG)	-0.008	-0.333	0.031	-0.246	0.813	0.000	1.000
head mass (mg)	0.827	-0.991	0.099	8.381	<0.001*	0.000	1.000
brain mass (mg)	0.543	-1.944	0.061	8.940	<0.001*	0.000	1.000
# facets	0.124	3.794	0.011	11.769	<0.001*	0.865	0.124
facet width	0.115	1.470	0.036	3.242	0.014*	0.000	1.000
interomm. angle (Φ)	0.022	.0595	0.040	0.553	0.597	0.227	0.761

Figure 1. Figure 1. Phylogenetic tree used for phylogenetic generalized least squares regression analysis (PGLS). The positions of each species on this tree were derived from Rasmussen & Cameron 2009.

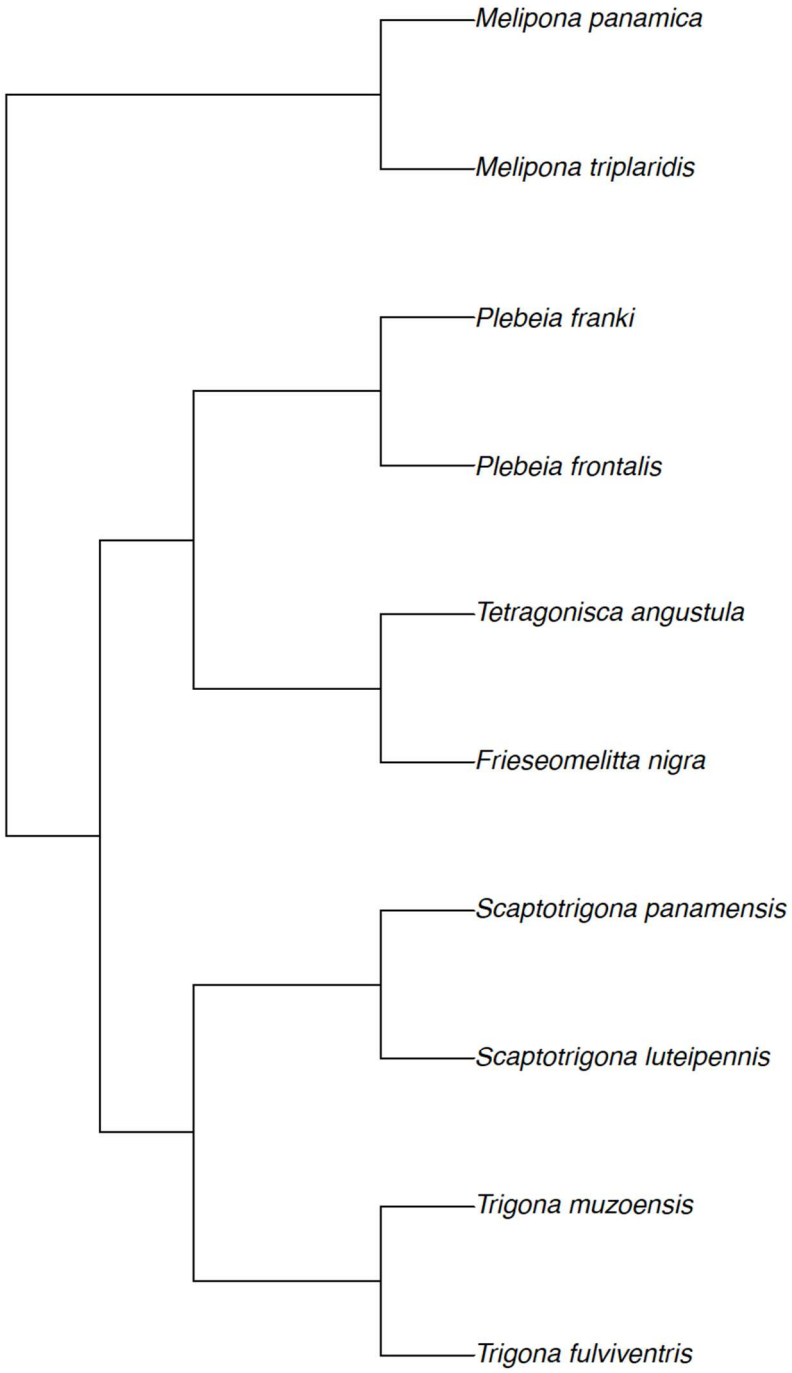


Figure 2. Diagram of the Y-maze used for testing visual differentiation and learning. Individual bees entered at the position of the arrow. An acrylic panel was put up behind it when it entered and that panel matched the white of the maze arms. Bees could see both patterns from the area inside the triangle indicated by dotted lines A choice was recorded when the bee crossed halfway into an arm of the maze.

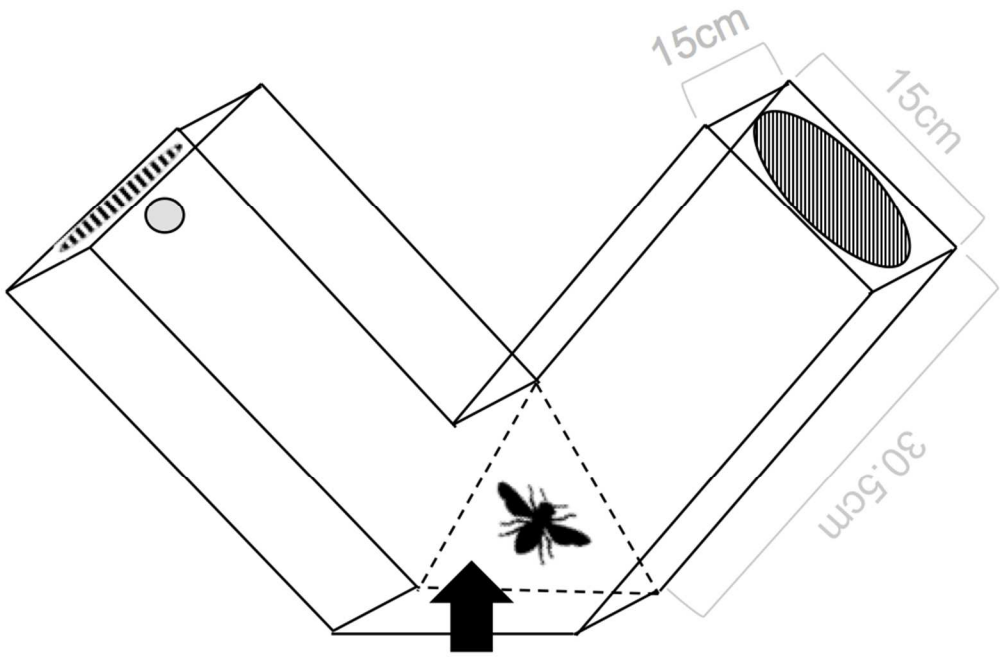


Figure 3. A) Frontal view of an eye of *M. panamica*. B) *Lateral view* of an eye with cutout demonstrating how ommatidia number and width were measured. C) Dorsal view of the eye showing how interommatidial angles were measured. Eye morphology measurements were performed in ImageJ after stacks of high-resolution macro photographs were compiled to create high-resolution images of the eyes of every species.

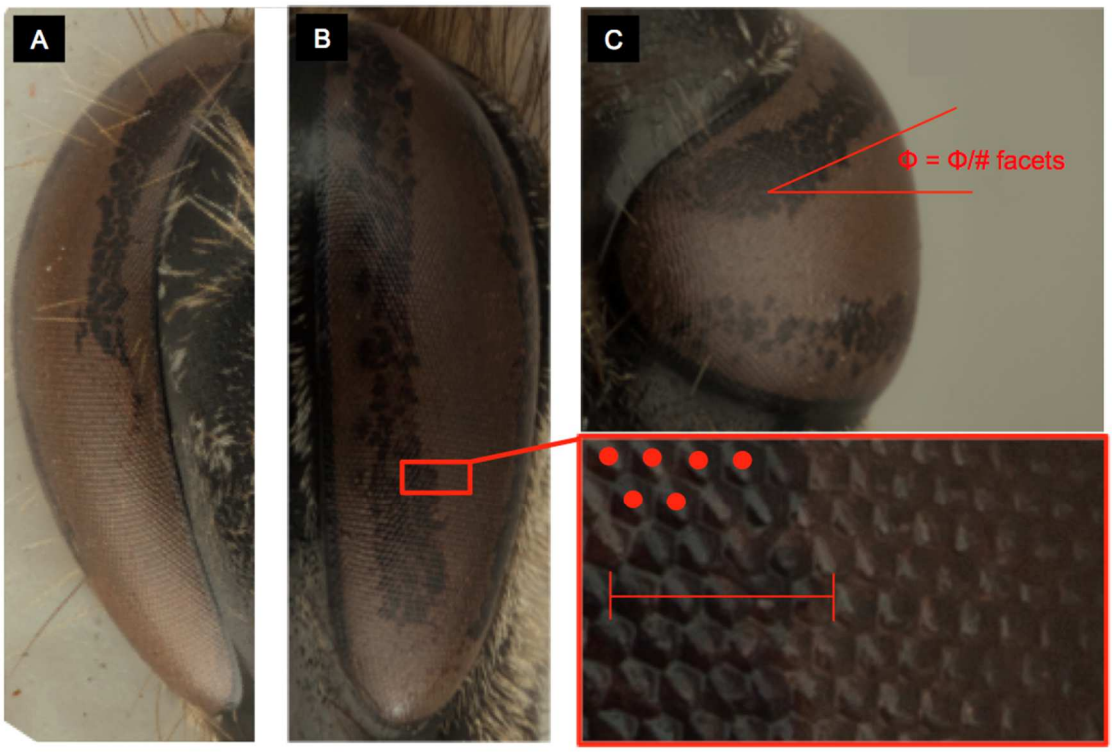


Figure 4. A) The mean proportion of correct choices was statistically the same across species of all sizes. B) The mean number of trials necessary to achieve 10 correct choices in a row was less, on average, in smaller stingless bee species (Table 3). C) Mean cumulative choice curves for all species in the learning test. Species are listed in order of size smallest to largest.

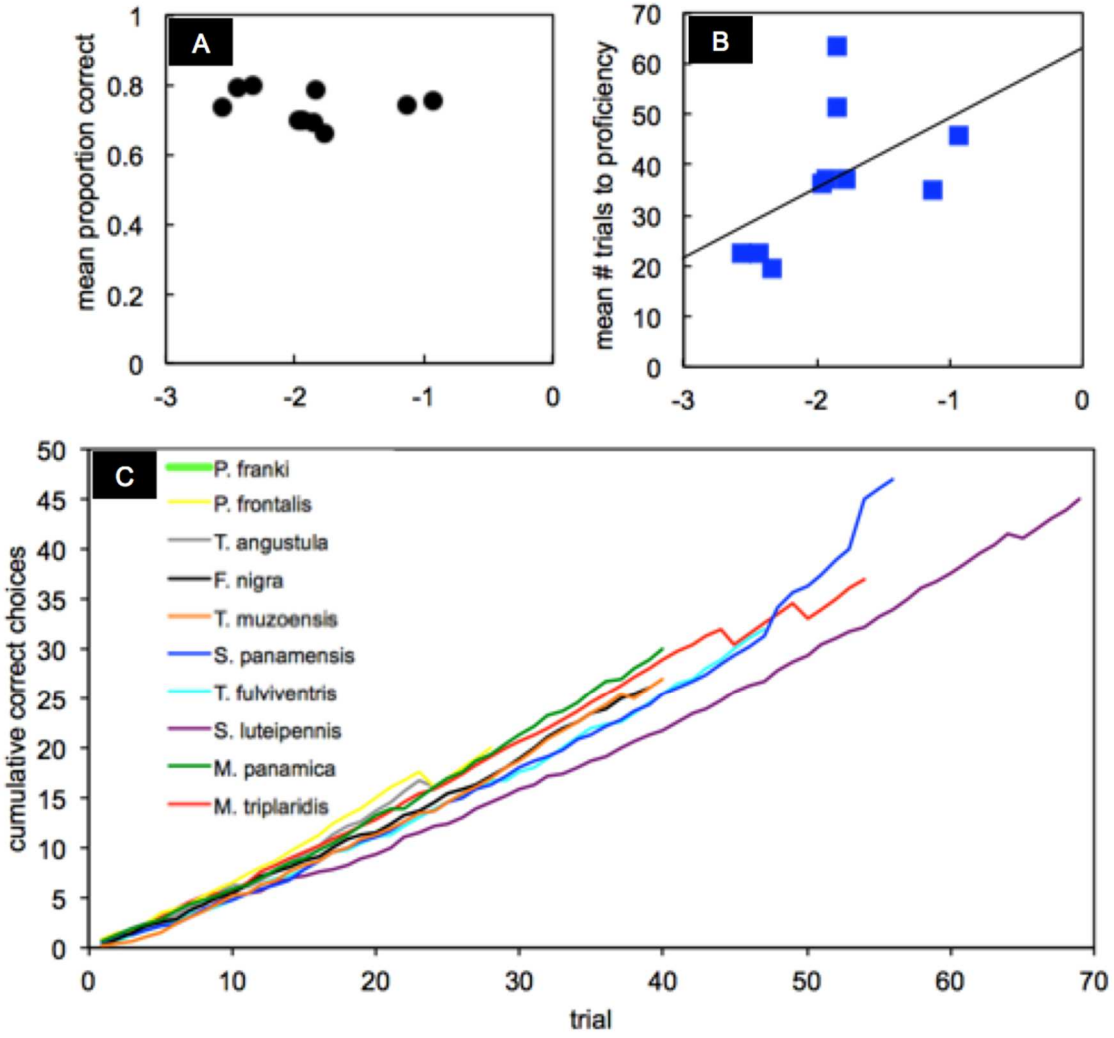


Figure 5. Figure 5. Body size did not affect the ability of stingless bees to distinguish patterns. A-F) plots of the log score of average correct number of choices out of ten trials vs. the average log masses for each species. Pattern pairs are shown on each panel.

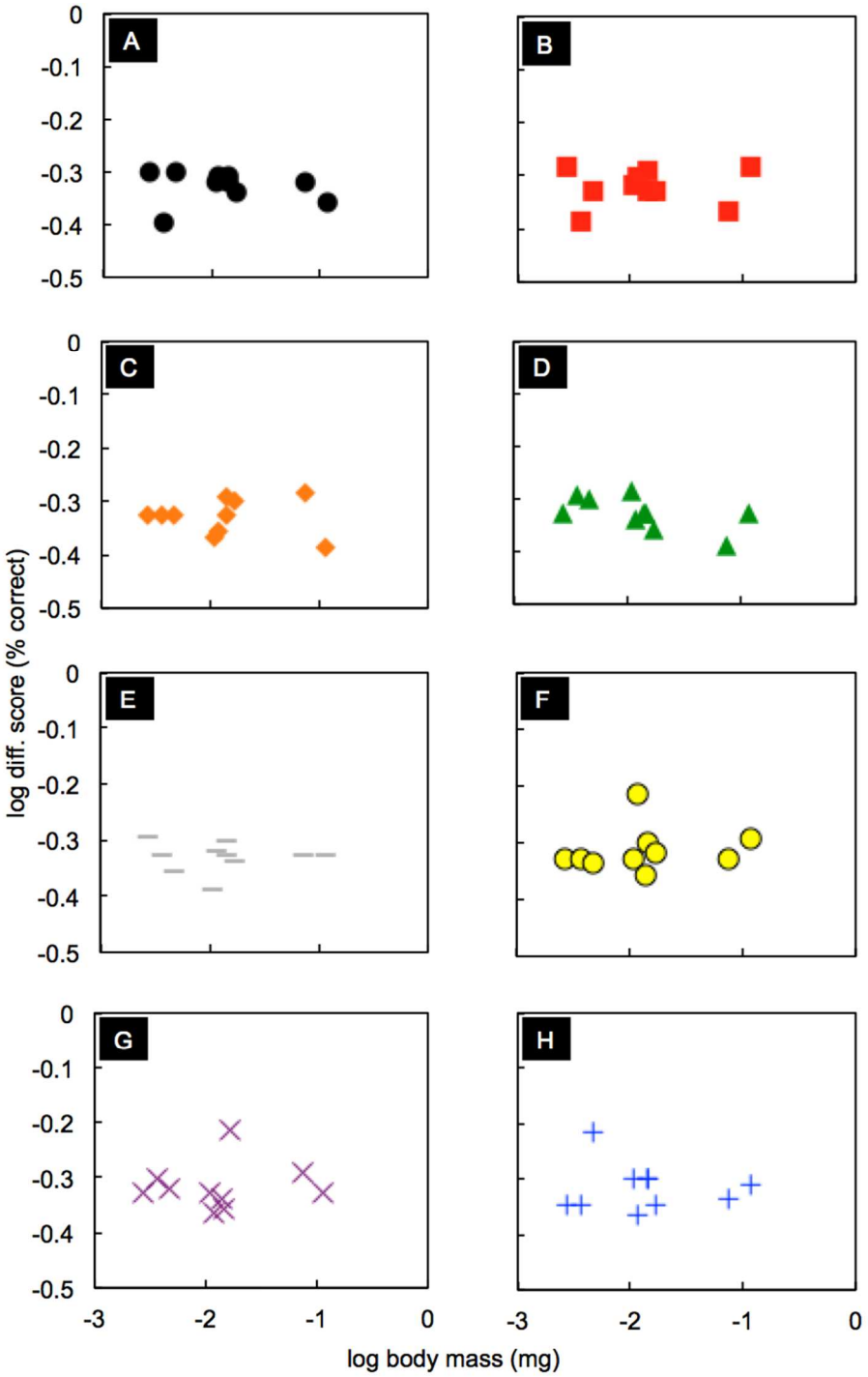
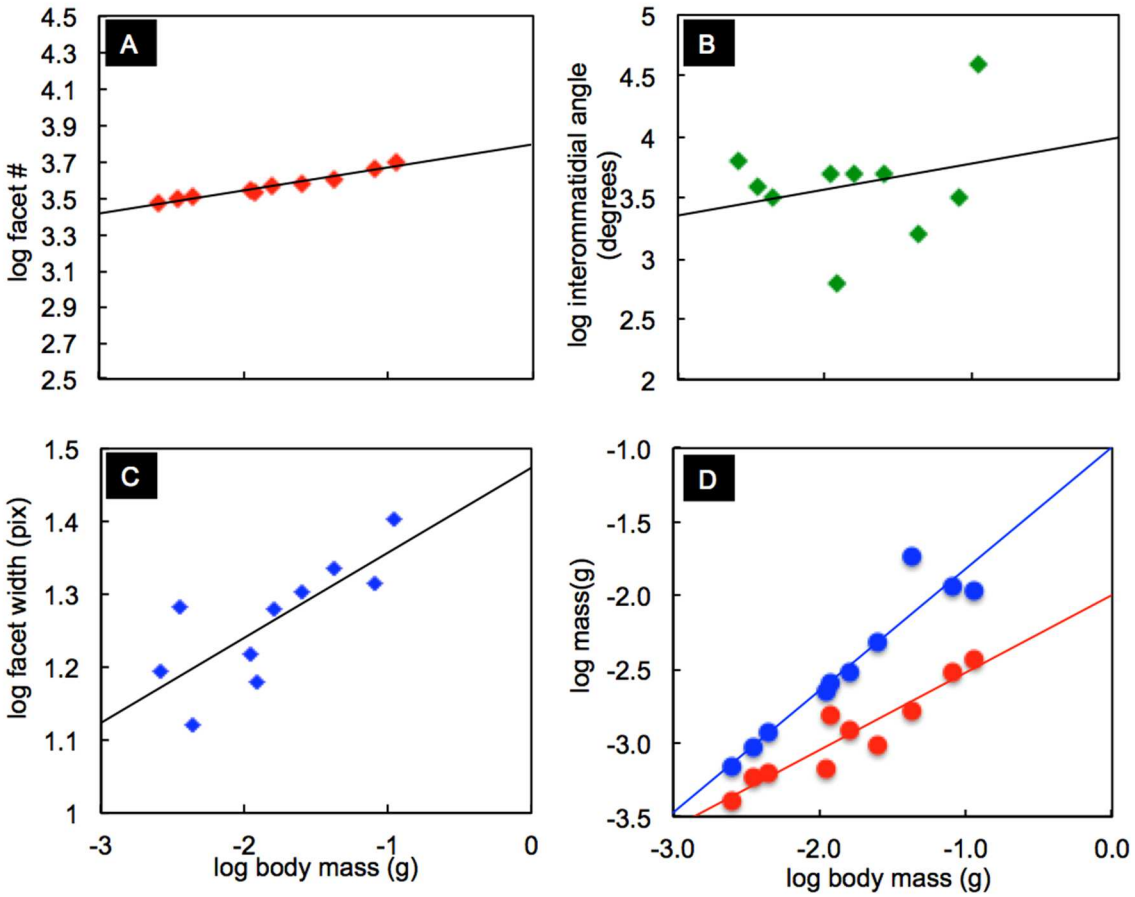


Figure 6. The scaling of A) facet number, B) interommatidial angles, C) facet width, and D) head (blue) and brain mass (red) in stingless bees. Smaller species had proportionally larger heads, brains, and eyes (facet counts, and facet widths) compared to larger species (results of PGLS analysis in Table 3).



REFERENCES

Barton, R.A., Harvey, P.H. 2000. Mosaic evolution of brain structure in mammals. *Nature* 405, 1055-1058.

Bonner, J. 1979. *Why Size Matters.* (Princeton University Press, Princeton, NJ. 2006).

Campi, K. L., Krubitzer, L. 2010. Comparative studies of diurnal and nocturnal rodents: differences in lifestyle result in alterations in cortical field size and number. *J. Comp. Neurol.* 518, 4491–4512.

Chapman, R. F. 1982. Chemoreception: the significance of receptor numbers. *Adv. Insect Physiol.* 16, 247–356.

Development and Evolution of Brain Size: Behavioral Implications. Edited by Hahn, M.E. et al. Academic Press Inc., New York. 1979.

Dusenbery, D. B. 1992. *Sensory Ecology.* W. H. Freeman & Co, New York, NY.

Eberhard, W., Wcislo, W. 2011. Grade changes in brain-body allometry: morphological and behavioural correlates of brain size in miniature spiders, insects and other invertebrates. *Adv. Insect Physiol.* 40, 155-213.

Eberhard, W.G. 2007. Miniaturized orb weaving spiders: behavioral precision is not limited by small size. *Proc. Roy. Soc. B* 274, 2203–2209.

Eberhard, W.G. 2011. Are smaller animals behaviorally limited? Lack of clear constraints in miniature spiders. *Anim. Behav.* 81, 813–823.

Galizia, C. G., Eisenhardt, D., Giurfa, M. (2012). *Honeybee Neurobiology and Behavior: A Tribute to Randolf Menzel*. New York: Springer Science & Business Media.

Gossette, R.L. 1969. Variation in magnitude of negative transfer on successive discrimination reversal (SDR) tasks across species. *Percept. Motor Skills* 29: 803-811.

Grebennikov, V.V. 2008. How small you can go: factors limiting body miniaturization in winged insects with a review of the pantropical genus Discheramocephalus and description of six new species of the smallest beetles (Pterygota: Coleoptera: Ptilii- dae). *Eur. J. Ent.* 105, 313–328.

Hanken, J. and Wake, D. B. 1993. Miniaturization of body size: organismal consequences and evolutionary significance. *Ann. Rev. Ecol. Syst*. 24, 501–519.

Harvey, P. H., Clutton-Brock, T. H. and Mace, G. M. 1980. Brain size and ecology in small mammals and primates. *Proc. Natl. Acad. Sci.* USA 77, 4387–4389.

Herculano-Houzel, S Mota, B., Lent, R. 2006. Cellular scaling rules for rodents. *Proc. Natl. Acad. Sci.* 103(32): 12138-12143.

Herculano-Houzel, S. 2009. The human brain in numbers: a linearly scaled up primate brain. *Frontiers Neurosci* 3(31):1-11.

Invertebrate Learning and Memory. 2013. Menzel R, Benjamin PR. Academic Press, Elsevier.

Isler, K. 2013. Brain Size Evolution: How Fish Pay for Being Smart. *Current Biol.* 23(2): R63-R65.

Iwaniuk, A. N., Nelson, J. E. and Whishaw, I. Q. 2000. The relationships between brain regions and forelimb dexterity in marsupials (Marsupialia): a comparative test of the principle of proper mass. Aust. J. Zoo. 48, 99–110.

Jander, U. and Jander, R. 2002. Allometry and resolution of bee eyes (Apoidea). *Arthropod Struct. Dev. 30*, 179–193.

Kaas, J. 2000. Why is brain size so important: design problems and solutions as neocortex gets bigger or smaller. *Brain Mind* 1:7-23

Kiltie, R. A. 2000. Scaling of visual acuity with body size in mammals and birds. *Funct. Ecol.* 14, 226–234

Kotrschal, A., Rogell, B., Bundsen, A., Svensson, B., Zajitschek, S., Brännström, I., Immler, S., Maklakov, A.A., Kolm, N. 2013. Artificial Selection on Relative Brain Size in the Guppy Reveals Costs and Benefits of Evolving a Larger Brain. *Current Biol.* 23(2): 168-171.

Land M. F., 1989. Variations in the structure and design of compound eyes, in Facets of Vision. Edited by Stavenga D.G., Hardle R.C. Pp 90-111.

Land M.F., Tatler B. W. 2009. *Looking and acting.* Oxford University Press, Oxford.

Land, M. F. and Nilsson, D. E. 2002. Animal Eyes. Oxford Animal Biology Series, Oxford.

Linke, R., Roth, G., Rottluff, B. 1986. Comparative studies on the eye morphology of lungless salamanders, family Plethodontidae, and the effect of miniaturization. 189(2): 131-143.

Matthews, R.W., Matthews, J. R. 2010. *Insect Behavior* (2nd ed.). New York: Springer Science & Business Media.

Milo, R, Jorgensen, P, Moran, U, Weber, G, Springer M. 2010. BioNumbers- the database of key numbers in molecular and cell biology. Nucleic Acids Research 38: D750–D753.

Niven, J. E. 2010. Nervous system evolution in relation to behavior. *Encyclopedia of Animal Behavior 2*, 527–533 (Oxford Academic Press, New York).

Niven, J., Farris, S. 2012. Miniaturization of nervous systems and neurons. *Current Biol.* 22, R323-R329.

Perl, C.D., Niven, J.E. 2016. Differential scaling within an insect compound eye. *Biol. Lett.* 12(3): 20160042. DOI: 10.1098/rsbl.2016.0042

Peters, R.H. 1983.*The Ecological Implications of Body Size*. (Cambridge University Press, New York).

Polilov, A. 2015. Small Is Beautiful: Features of the Smallest Insects and Limits to Miniaturization. *Annu. Rev. Entomol.* 6:103-121.

Rensch, B. Histological change with evolutionary changes of body size. *Evolution* 2, 218–230 (1948).

Revell, L.J. 2010. Phylogenetic signal and linear regression on species data. *Methods Ecol. Evol*. 1: 319–329.

Riddell, W., Corl, K., Van Dyke, B., Reimers R.O. 1977. Discrimination learning differences and similarities as a function of brain index. *Physiol Behav,* 13(3): 401-405.

Rossel, S. 1980. Foveal fixation and tracking in the praying mantis. *J. Comp. Physiol. A.* 139:307-331.

Roth, B.G., Blanke, J., Ohle, M. 1995. Brain Size and Morphology in Miniaturized Plethodontid Salamanders. *Brain Behav. Evol.* 45:84–95.

Rutowski, R. L. 2000. Variation of eye size in butterflies: inter- and intraspecific patterns. *J. Zool.* Lond 252, 187–195.

Spaethe, J. and Brockmann, A. 2007. Size determines antennal sensitivity and behavioral threshold to odors in bumblebee workers. *Naturwissenschaften* 733–739.

Spaethe, J. and Chittka, L. 2003. Interindividual variation of eye optics and single object resolution in bumblebees. *J. Expt. Biol.* 206, 3447–3453.

Zhang, S. W., Bartsch, K., & Srinivasan, M. V. 1996. Maze learning by honeybees. *Neurobiology of learning and memory*, *66*(3), 267–82. doi:10.1006/nlme.1996.0069

CHAPTER 3

SIZE-DEPENDENT SCALING OF INSECT FLIGHT METABOLISM REVEALS AN ENERGETIC BENEFIT OF BEING SMALL

ABSTRACT

Understanding the effect of body size on flight costs is critical for development of models of aerodynamics and animal energetics. Prior scaling studies that have lacked animals in the 6-20 mg size range have shown that flight costs scale hypometrically in insects and birds, but also that metabolic rates of smaller insects (> 10 mg) are lower and disjunct from those of larger animals. I studied the flight physiology of 13 stingless bee species (1-115 mg), filling in this key gap. Metabolic rate during hovering of stingless bees scaled hypermetrically (scaling slope = 2.11). Synthesizing across all flying insects, I demonstrate that the scaling of flight metabolic rate changes from hypermetric (slope = 1.2) to hypometric (slope = 0.67) at approximately 53 mg body mass. Reduced metabolic flight costs likely provide a selective advantage for the evolution of small body size among flying insects.

MAIN TEXT

Understanding how body size affects animal function is one of the central themes of biology; such scaling studies have provided key syntheses of organismal function and macroecology.[1] Flight is a key trait for the evolutionary success of insects and birds, being integral to resource collection (pollination), migration and defense. The scaling of flight metabolic rate with mass in insects remains a controversial issue. Studies of hovering moths and bees ranging in mass from 100-1100 mg have shown that flight metabolic rates scale hypometrically with slopes of log metabolic rate on log mass of 0.63-0.77, with wing beat frequencies consistently shown to decline in larger insects *(9-11).* In contrast, a meta-analysis by Niven and Scharlemann (2005) suggested that across all insects, flight metabolic rates scale hypermetrically with mass$^{1.1}$, and that this was due to insects below 10 mg in mass having distinctly lower flight metabolic rates than insects above this size *(12).* However, these authors noted that their conclusions were hampered by a dearth of studies of insect flight in the size classes across which flight costs seem to change dramatically (6-20 mg).

I measured flight metabolic rates in 13 species of stingless bees with body masses ranging from 1.5-115 mg. I also measured body temperature in flight, wing beat frequency, voluntary maximal load carriage, and wing and body size, all important factors that determine overall flight performance. Stingless bees (Meliponini) are an outstanding taxon for examination of the effects of body size on flight physiology across a size range of approximately 1-150 mg (Figs. 1A, S1) with a fairly well-defined molecular phylogeny *(13-15).* Some lineages, especially the genus *Melipona*, have large

species ranging in body mass up to 150 mg *(16)*. Miniaturization has evolved multiple times among 33 genera *(17,18)* and it is thought that ancestral meliponines were moderately sized, perhaps 50 mg *(13,16,17)*. The smallest species I used, *Trigonisca buoyssoni,* was 1.5 mg in size while the largest, *Melipona triplaridis*, was 115 ± 5mg.

Flight metabolic rates scaled hypermetrically across stingless bee species, with a scaling exponent of 2.25 (Fig. 1B, Adj. R^2=0.66, P<0.001, λ=0.00, P= 0.0175). This slope was not significantly affected by corrections using phylogenetically generalized least squares analysis (PGLS, Table S1). The 95% confidence limits for this slope did not include isometry (slope = 1) or the hypometric exponents found for euglossine bees (commonly known as orchid bees), moths, or other flying insects *(10,19,20)*. The different scaling patterns in euglossines and stingless bees are not due to differences in absolute cost, since flight metabolic rates are similar at body masses at which both taxa have been measured (circa 100 mg [Fig. 2]).

Flight muscles of insects generate substantial heat, and most insects larger than 50 mg fly at thorax temperatures 5-20°C above air temperature, while insects with body masses lower than 50 mg have high cooling rates and usually have body temperatures close to air temperature *(21-23)*. Might the hypermetric scaling of hovering metabolic rates be explained by changing flight muscle temperatures with size? To test how size affected the temperatures of the thorax, head and abdomen, I used a "grab and stab" technique using a high-speed thermal probe, capturing and measuring temperatures within 1-3 sec of flight by stabbing the bees through a plastic bag in which they were hovering (see Supplemental Methods). To test how thorax temperature affected flight

metabolic rates, I also flew bees from all thirteen species at a range of air temperatures between 25-45°C while measuring metabolic rate and took thorax temperatures using the same grab and stab method to generate a thermal performance curve and calculate Q_{10}. Body temperatures of stingless bees demonstrated the size-related pattern expected from studies of other insects (Fig. 1C). Stingless bees heavier than 70 mg (*M. panamica* and *M triplaridis*) had substantially elevated body temperatures during hovering in the metabolic chambers, more than 10°C above air temperature, as previously shown *(24).* In these two large species, thorax temperatures were the highest, as predicted by heat production in the flight muscles, and the abdomen was the coolest region (Fig. 1C). In contrast, in stingless bee species less than 20 mg, head, thorax, and abdomen temperatures were fairly uniform and only about 1-3°C above air temperature during hovering (Fig. 1C).

Can the difference in thorax temperatures of large and small stingless bees during hovering explain the hypermetric scaling of flight metabolic rates in stingless bees? The metabolic rates of large flying insects can increase *(25,26),* decrease *(27-31),* or be independent of thorax temperature *(32-36).* However, for the small insects that have been previously measured (primarily Dipterans), flight performance does increase strongly over cool to moderate ranges of thoracic and air temperature, shown by Q_{10} values for wing beat frequency, flight speed, force production, power output, and metabolic rates of 1.2 - 2 *(38-41).* To test the possibility that the lack of hypometric scaling of stingless bee flight metabolic rate was caused by the variation in flight muscle temperatures across species, I fit the thorax temperature data with a third-order polynomial line of best fit (Fig. 1D), and then used this function and a Q_{10} of 2 to predict thermally-corrected flight

metabolic rates for each species (Fig. S2). The scaling slope of the temperature-corrected flight metabolic rates was still significantly hypermetric with a slope of 2.11 (Fig. S2). Additionally, I measured the effect of temperature on flight metabolic rates for one species of stingless bee, *Scaptotrigona luteipinnis.* For this species, flight metabolic rate was relatively constant at thorax temperatures of 25-40ºC, and then declined at higher thorax temperatures (Fig. 1D). Thus the hypometric scaling of flight metabolic rates in stingless bees cannot be explained by thermal variation across size.

The differential scaling of flight metabolic rates in stingless bees and euglossine bees is associated with differential scaling of their wing morphology. Larger stingless bees had relatively smaller wings, as the slope of total wing area scaled with body mass with a scaling exponent of 0.56 (Fig. 1E, Table S1), significantly less than the isometric prediction of 0.67, and contrasting with the pattern for euglossine bees, in which larger bees have relatively larger wings than predicted by isometry (44). The relatively smaller wing area in larger bees is because these wings are relatively narrower, as wing lengths scaled isometrically (Fig. S3, Table S1). One possibility is that the relatively larger wings in smaller bees could create more lift per stroke, potentially reducing energetic cost and contributing to the lower flight cost per gram observed in smaller bees.

In contrast to the scaling of wing area, the masses of body segments scaled similarly to other insects. Stingless bee thorax mass scaled about isometrically (Table S1, slope= 1.109 ± 9.140 SE, Adj. R^2=0.74 λ=1.0), consistent with orchid bees (probably the most studied group of for flight physiology) and other bees and insects measured *(11,19).* Abdomen mass also scaled isometrically (slope= 1.046±0.113 SE , Adj. R^2= 0.7000,

λ=0.976). Neither had slopes significantly different from 1 (p(thorax)= 0.69, p(abdomen)= 0.46) head mass scaled hypometrically (slope= 0.86, Adj. R^2= 0.7973, λ=1.00) as found for other insects and vertebrates (45).

In contrast to the general finding of declining wing beat frequency with increased body size in larger insects (e.g. for euglossines, the scaling exponent for wing beat frequency is -0.31 *(29))*, wing beat frequencies of stingless bees were independent of mass (Fig. 1E, Fig. S4). This finding is supported by Byrne (1988), who demonstrated that wing beat frequencies are independent of size in aphids and white flies less than 30 mg.

As for most other fliers studied (45), all bees lifted similar fractions (about 20%) of their body mass during voluntary load-lifting of nectar, despite their varied thorax temperatures and hypermetric scaling of costs of flight when not loaded (Fig. 1E, Table S1). Similarly, using a progressive load-lifting method, Dillon and Dudley found that vertical force production scaled either isometrically (using log-transformed data) or hypometrically (using raw data) across Euglossine bees. Thus smaller stingless bee species can carry similar loads (mass-specific) at reduced energetic cost relative to larger stingless bee species.

I combined our data with all currently published data on flight metabolic rates of hovering insects to synthesize the scaling of flight costs across this clade. Flight metabolic rates were corrected to watts using published respiratory quotients for the species or related species (Table S2). The flight metabolic rates of stingless bees closely approximated costs of other similarly sized insects (Fig. 2). Inspection of all insect flight

metabolic rates indicated that there was a breakpoint in the scaling of metabolic rates with size. A breakpoint analysis indicated that the breakpoint occurred at 33 mg (Fig. 2). A biphasic model using two size classes (above and below 33 mg) better explained the scaling of metabolic rates than a simple continuous log-log model, based on residual MSE of the generated breakpoint models compared to the standard model (Table S3). I next fit linear models to log-log plots of metabolic rates vs. mass above and below 42 mg; these had high r^2 values, particularly in the low-mass range, (Table S3). The scaling slope of flight metabolic rate below 33 mg was 1.199, and 0.675 above 33 mg (Fig. 2). Thus I conclude that scaling of insect flight metabolic rates is biphasic, with hypermetric scaling in the low range and hypometric scaling in the high range.

The mechanisms responsible for the biphasic scaling of flight costs remain unclear, but likely include both aerodynamic and evolutionary mechanisms (12). Aerodynamic costs of flight may be reduced among smaller insects, partly due to performance at low Reynolds numbers. As body mass decreases, viscosity gradually dominates over inertial forces. Marden and Allen (2002) and Marden (2005) predicted, with a sample size of four small insect species, that the mass-scaling exponent of force production in flight should gradually decline with body size, consistent with hypermetric scaling of flight costs *(48,49)*. Evolutionary mechanisms include the finding that smaller stingless bees have relatively larger wings (Figs. 1E, S2, Table S1), as well as decreased venation on the laminar surface of the forewing, a relatively larger stigma, and a heavier forewing leading edge (53), all potentially providing greater lift generation without increased energy expenditure. Smaller stingless bees also have proportionally larger

heads; this contributes to a shift in the center of mass to a more forward position (54,55). Such morphological changes may contribute to use of different aerodynamic mechanisms pf force production. Changes in wing stroke, pitch, roll, and/or yaw could result in sufficient energy savings (56-57). Regardless of the mechanism, the reduced cost of flight in smaller insects will likely reduce costs of foraging, defense and migration, providing a significant selective advantage for the evolution of small body size among insects.

METHODS

Study sites and stingless bee collection

Stingless bee foragers from 13 species (*Melipona triplaridis*, *Melipona panamica*, *Scaptotrigona panamensis*, *Scaptotrigona luteipinnis*, *Trigona fulviventris*, *Trigona muzoensis*, *Tetragonisca angustula*, *Frieseomelitta nigra*, *Lestrimelitta danuncia*, *Plebeia franki*, and *Plebeia frontalis*) were captured returning to nests at several locations in the Republic of Panamá. *S lutipinnis*, *T. angustula*, *F. nigra*, and *T. fulviventris* were captured in Gamboa, Panamá while *T. muzoensis*, and *Plebeia frontalis* were collected on Barro Colorado Island. *M. triplaridis*, *S. panamensis*, and *L. danuncia* were collected from the property of David Roubik in Curundu, Panamá and *P. franki* and *M. panamica* were captured at the Santa Rita Arriba property of David Roubik. In each case, foragers were identified and captured as they returned to the nest from a single colony of each species. *Trigonisca atomaria* and *Trigonisca buoyssoni* were collected while foraging at flowers using the canopy crane at Parque Naturál Metropolitano, Panama City, Panamá, and at Santa Rita Arriba while foraging on honey water. Individuals were placed in vials with sugar water for food if they could not be measured within one hour of capture. All bees were brought back to the Smithsonian Tropical Research Institute lab in Gamboa, Panamá for measurement.

Respirometry and wing beat frequency analysis.

I used flow-through respirometry and measured CO_2 emission and flow rate. Ambient air was pushed through silica and soda lime scrubber columns by an aquarium

pump, and flow rate was adjusted using a Sable Systems FlowBar 8 mass-flow controller (resolution ±0.1 ml/min below 100 ml min^{-1}; resolution 1 ml min^{-1} above 100 ml min^{-1} flow rate). Excurrent CO_2 was measured using a LiCor 6252 plumbed in the differential mode (the reference cell measured the air flowing into the chamber and the sample cell measured air flowing out of the chamber; resolution was approximately 0.2 ppm with hardware and software time-averaging of 1 sec). The system was calibrated and spanned using a CO_2 tank containing 1221 ppm CO_2 (as measured by by J. Shik with a LiCor 7000 calibrated against a certified span gas), with the zero and span recalibrated each time the flow rate was changed, and zeroed before and after each bee was measured. We used four different cylindrical glass flight chambers with volumes of 15ml, 70ml, 150ml, and 550ml; chamber sizes were adjusted to the size of the bee. We chose the smallest chamber that a species would fly consistently in. Flow rates were adjusted to chamber size so that the 95% washout time for that chamber was not less than 45 sec; flow rates ranged from 150 ml min^{-1} for the smallest chambers to 1000 ml min^{-1} for the largest chamber used. CO_2 levels during flight ranged from 6ppm to 175ppm, with a minimal signal-to-noise ratio of 10. The analog outputs of the CO_2 analyzer and mass flow-controller were digitized and recorded with a Sable Systems UI-2 and a computer using Expedata Pro 1.7.2 (digitization resolution was 0.15 ppm for the CO_2 analyzer and 0.1 ml min^{-1} for the mass flow-controller).

Flight behavior included constant hovering, erratic flight in which the bee collided with the chamber walls, and flight in which the bees flew with their legs attempting to gain purchase on the Fluon-coated glass walls. Several methods were used to maintain

good flight behavior including agitation of the chamber and shining a bright light above the chamber while its surroundings were kept dark. We only accepted data from bees that exhibited at least 30 sec of consistent flight behavior that was accompanied by a relatively high and consistent CO_2 reading measured after the 45 sec required for washout of any atmospheric CO_2 that may have entered the chamber when the bee was placed into it. The average flight duration we measured was 43 sec. After measuring CO_2 emission during flight, the air pump was turned off and we inserted a Sony ECM-PC60 mini electret condenser microphone to record wing beat frequency for each bee. This was recorded and analyzed using Raven Lite 1.0 software. A subsample of 4-5 bees/species were then stimulated to fly in the same chambers and filmed with a MotionPro X high-speed video camera at 1000frames/sec to verify wing beat frequency data acquired with a microphone. The average wing beat frequency from three measures per individual was used for analysis.

Body temperature in flight

After measurements of wing beat frequency, bees were removed from the chamber and placed in a plastic Ziploc bag; they continued flying within the bag until measurement of body temperature was accomplished. We used a 'grab-and-stab' measurement technique (Roberts & Harrison 1999) with a Physitemp MT-29/1 hypodermic needle microprobe (29 ga, 0.025sec time constant) and a Physitemp BAT-10 thermocouple meter. To minimize thermal transfer from human to bee, we wore insulated gloves to hold the temperature microprobe, held the probe at least an inch away from the

measuring tip, and restrained bees by pulling the plastic bag tight about them, on top of a thick Styrofoam board. We also tested that heat transfer from the thermocouple to the bees and found that it was negligible. First we calibrated the thermocouples to a known temperature of 0°C in an ice water slurry. We equilibrated dead bees of various sizes to air temperature before measuring them and air with the thermocouple probe. All bees were within ±0.5°C of air temperature. An additional 5 bees per species were killed by freezing for one hour and we measured their body segment temperatures after keeping them at room temperature for 15 minutes, the length of time needed for body temperatures to stabilize; these measurements verified that dead bees measured with this technique had body segment temperatures within 0.4°C of the measured air temperature. Dead bees were also warmed to various temperatures (28°C, 30°C, and 32°C) to check that they matched the surrounding air temperature.

Air, abdomen, and head temperatures were measured in random order for every bee after first measuring thorax temperature; thorax temperatures were taken within 1 sec of restraint and all temperatures were measured within an additional 2 sec. Finally, we determined masses for each bee.

Wing morphology and load carriage.

We removed the wings for 10 individuals per species and flattened them onto white cardstock paper with transparent tape. A digital image of each wing was taken with a 1mm grid for calibrating measurements. Area measurements were performed in ImageJ. To determine load carriage, we starved 10 bees per species for 2 hours, then fed them

50% sucrose solution to satiation and encouraged them to fly. Each bee was weighed before eating and immediately after take-off.

Phylogenetic and Statistical Analysis.

All data for stingless bees are represented as species means ± SE (standard error) of individual measurements. The effect of body mass was tested using least squares linear regression performed on log-transformed data to obtain the metabolic rate equation aM^b where a=y-intercept, M=body mass, and b= allometric scaling coefficient (Darveau et al. 2005). We converted metabolic rates (ml g^{-1} h^{-1}) to watts assuming RQ=1 based available data for hymenopterans (Suarez et al 2005) and because bees were fed solely on a diet of sucrose water while in captivity. Further analyses of wing beat frequency, wing area, wing loading, and flight body temperature were performed using Phylogenetic generalized least squares regressions (PGLS) in R on log-transformed data. A comprehensive maximum likelihood tree based on Rasmussen & Cameron (2010) was adapted for this study by pruning unnecessary species and adding species, which did not appear on the published phylogenies. Branch lengths for all tip species were then set equal to one (Rasmussen & Cameron 2010, Rasmussen & Cameron 2007, Garamzsegi 2014). PGLS was performed for all analyses using all statistically possible tree topologies and results were obtained using the topology with the highest likelihood (Garamzsegi 2014).

We compared the known metabolic rates of 117 flying insects by compiling literature values (Table S2) and converting metabolic rates to watts. When RQs were

available, they were incorporated into the metabolic rate equation or assumed to be 1. Data points were eliminated if they did not use modern methods (flow- through or stop-flow respirometry or precise gas isotope studies) for determining flight metabolic rates or were measured in non-standard conditions, such as fluctuating temperature, humidity, air pressure or air flow. Breakpoint models of log body mass vs. log metabolic rate were generated in R using the breakpoints and lm.br packages (Priyadarshana W.J.R.M. 2016). The model was unconstrained to allow discontinuous slopes on either side of breakpoints and bootstrap restart sampling between 20-60mg body mass. This generates multiple possible piece-wise regressions which differ in slope and breakpoint. We chose the regression with the lowest error represented as AKC. We compared this piece-wise regression to the standard model with a continuous slope across body size of 0.75 using AIC comparisons included in the breakpoints package in R.

TABLES & FIGURES

Table S1. Phylogenetic generalized least squares regression (PGLS) statistics for all physiological variables. All scaling data were regressed using PGLS as part of the regression model, which integrates linear models to fit a line based on evolutionary relatedness through data. Phylogenetic signal (λ) is on a 0-1 scale where 1 is the highest amount of signal possible. Coefficient t measures the distance of the line estimate (slope) from zero, with higher number demonstrating higher significance of the relationship between variables (body mass and the physiological variables shown).

physiological variable	slope	intercept	t	p	adj. r^2	λ	st. err
flight MR (CO_2 ml/h)	2.234	2.843	14.466	1.668e-08*	0.946	0.000	0.154
head temperature(°C)	0.225	0.804	1.8284	0.095	0.163	0.490	0.123
thorax temperature (°C)	0.298	1.043	2.4631	0.032*	0.297	1.000	0.121
abdomen temperature (°C)	0.146	0.578	1.3222	0.213	0.059	0.570	0.111
wing beat frequency (Hz)	0.015	2.323	0.4303	0.675	-0.073	0.000	0.035
load carriage (mg)	0.869	0.776	15.66	7.693e-08*	0.967	0.000	0.056
total wing area (mm^2)	0.499	0.431	8.749	2.762e-06*	0.863	0.898	0.067
forewing area (mm^2)	0.567	1.869	8.308	4.55e-06*	0.850	0.832	0.068
hindwing area (mm^2)	0.643	1.639	9.757	9.449e-07*	0.887	1.000	0.066
forewing width (mm)	0.269	0.783	8.234	4.963e-06*	0.848	0.860	0.033
forewing length (mm)	0.301	1.281	7.767	8.646e-06*	0.832	0.842	0.039
hindwing length (mm)	0.314	1.147	8.459	3.826e-06*	0.855	0.775	0.037
hindwing width (mm)	0.315	0.585	8.110	5.733e-06*	0.844	0.904	0.039
head mass (mg)	0.860	-0.921	9.839	4.097e-06*	0.906	0.000	0.087
thorax mass (mg)	1.046	-0.235	9.2753	6.668e-06	0.895	0.858	0.113
abdomen mass (mg)	1.109	-0.485	7.9065	2.431e-05	0.860	0.567	0.140

Table S2. Insects used to compare flight metabolic rates across flying insect species in order of classification with masses, flight metabolic rates, and references used for mining the data.

Order	Family	Species	References	Mass (g)	Flight Met. Rate (w)
Coleoptera	Cerambycidae	*Phorocantha semipunctata*	Chappel & Rogowitz 2000	0.3150	0.0245
Coleoptera	Scarabaeidae	*Cotinus mutabilis*	Josephson *et al.* 2001	1.2000	0.8639
Coleoptera	Scarabaeidae	*Cotinus texana*	Chappell 1984	1.2900	0.4450
Coleoptera	Scarabaeidae	*Mecynorrhina savagei*	Klok, J. unpublished	5.5063	0.8918
Coleoptera	Scarabaeidae	*Pachnoda sinuata*	Auerswald et al. 1998	1.0000	0.0074
Dictyoptera	Blattidae	*Periplaneta americana*	Niven Scharlemann 2005'	1.2053	0.1527
Diptera	Calliphoridae	*Lucilia sericata*	Niven Scharlemann 2005'	0.0326	0.0134
Diptera	Culicidae	*Aedes flavescens*	Niven Scharlemann 2005'	0.0032	0.0006
Diptera	Culicidae	*Aedes nearcticus*	Niven Scharlemann 2005'	0.0058	0.0011
Diptera	Drosophilidae	*Drosophila melanogaster*	Heymann & Lehmann 2006, Lehmann & Schutzner 2009, Lehmann 2001, Niven & Scharlemann 2005	0.0010	0.0001
Diptera	Drosophilidae	*Drosophila mimica*	Niven & Scharlemann 2005'	0.0028	0.0004
Diptera	Drosophilidae	*Drosophila nikananu*	Niven & Scharlemann 2005'	0.0006	0.0001
Diptera	Drosophilidae	*Drosophila virilis*	Niven & Scharlemann 2005'	0.0014	0.0001
Hemiptera	Cicadidae	*Fidicina mannifera*	Bartholomew & Barnhart 1984	2.8380	0.6471
Hymenoptera	Apidae	*Apis mellifera*	Niven & Scharlemann 2005'	0.0979	0.0252
Hymenoptera	Apidae	*Bombus edwardsii*	Niven & Scharlemann 2005'	0.4000	0.1832
Hymenoptera	Apidae	*Bombus lucorum*	Niven & Scharlemann 2005'	0.5113	0.0913
Hymenoptera	Apidae	*Bombus terrestris*	Darveau *et al.* 2014, Hedenstrom *et al.* 2001	0.1678	0.0916

Hymenoptera	Apidae	*Eufriesa spp.*	Darvaeau et al. 2005'	0.4000	0.1644
Hymenoptera	Apidae	*Eufriesia pulchra*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.3879	0.2036
Hymenoptera	Apidae	*Euglossa bursigera.*	Darvaeau *et al.* 2005'	0.0840	0.0473
Hymenoptera	Apidae	*Euglossa championi*	Darvaeau *et al.* 2005'	0.1360	0.0519
Hymenoptera	Apidae	*Euglossa cognata*	Darvaeau *et al.* 2005'	0.1590	0.0831
Hymenoptera	Apidae	*Euglossa crassipunctata*	Darvaeau *et al.* 2005'	0.0670	0.0315
Hymenoptera	Apidae	*Euglossa despecta*	Darvaeau *et al.* 2005'	0.1120	0.0572
Hymenoptera	Apidae	*Euglossa dissimula*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.1020	0.0625
Hymenoptera	Apidae	*Euglossa hansoni*	Darvaeau *et al.* 2005'	0.0820	0.0549
Hymenoptera	Apidae	*Euglossa heterosticta*	Darvaeau *et al.* 2005'	0.0640	0.0357
Hymenoptera	Apidae	*Euglossa imperialis*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.1727	0.0974
Hymenoptera	Apidae	*Euglossa mandibularis*	Casey *et al.* 1985	0.9025	0.0986
Hymenoptera	Apidae	*Euglossa mixta*	Darvaeau *et al.* 2005'	0.0940	0.0568
Hymenoptera	Apidae	*Euglossa saphirina*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.0630	0.0409
Hymenoptera	Apidae	*Euglossa spp.*	Darvaeau *et al.* 2005'	0.1000	0.0587
Hymenoptera	Apidae	*Euglossa tridentata.*	Darvaeau *et al.* 2005'	0.1100	0.0652
Hymenoptera	Apidae	*Eulaema bombiformis*	Darvaeau *et al.* 2005'	0.9830	0.4847
Hymenoptera	Apidae	*Eulaema cingulata*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.5454	0.2323
Hymenoptera	Apidae	*Eulaema meriana*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.9077	0.2876
Hymenoptera	Apidae	*Eulaema nigrita*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.4198	0.2217
Hymenoptera	Apidae	*Eulaema spp.*	Darvaeau *et al.* 2005'	0.8000	0.1761

Hymenoptera	Apidae	*Exaerete frontalis*	Casey *et al.* 1985, Darvaeau *et al.* 2005'	0.6716	0.2023
Hymenoptera	Apidae	*Exaerete spp.*	Darvaeau *et al.* 2005'	0.8000	0.1526
Hymenoptera	Apidae	*Frieseomelitta nigra*	(this publication)	0.0108	0.0023
Hymenoptera	Apidae	*Lestrimelitta danuncia*	(this publication)	0.0096	0.0020
Hymenoptera	Apidae	*Melipona panamica*	(this publication)	0.0734	0.0323
Hymenoptera	Apidae	*Melipona triplaridis*	(this publication)	0.1157	0.0483
Hymenoptera	Apidae	*Plebeia franki*	(this publication)	0.0027	0.0006
Hymenoptera	Apidae	*Plebeia frontalis*	(this publication)	0.0037	0.0007
Hymenoptera	Apidae	*Scaptotrigona lutipinnis*	(this publication)	0.0144	0.0037
Hymenoptera	Apidae	*Scaptotrigona panamensis*	(this publication)	0.0141	0.0037
Hymenoptera	Apidae	*Tetragonisca angustula*	(this publication)	0.0047	0.0012
Hymenoptera	Apidae	*Trigona fulviventris*	(this publication)	0.0168	0.0032
Hymenoptera	Apidae	*Trigona muzoensis*	(this publication)	0.0117	0.0016
Hymenoptera	Apidae	*Trigonosca atomaria*	(this publication)	0.0018	0.0003
Hymenoptera	Apidae	*Trigonosca bouyssoni*	(this publication)	0.0015	0.0001
Hymenoptera	Apidae	*Xylocopa californica*	Niven & Scharlemann 2005'	0.6000	0.2219
Hymenoptera	Apidae	*Xylocopa capensis*	Niven & Scharlemann 2005'	1.2000	0.3734
Hymenoptera	Megachilidae	*Megachile rotundata*	Bennett *et al.* 2013, 2014	0.0335	0.0025
Hymenoptera	Pteromalidae	*Nasonia giraulti*	Lehmann & Heymann 2006'	0.0004	0.0003
Hymenoptera	Pteromalidae	*Nasonia longicornis*	Lehmann & Heymann 2006'	0.0006	0.0003
Hymenoptera	Pteromalidae	*Nasonia vitripennis*	Lehmann & Heymann 2006'	0.0005	0.0004
Lepidoptera	Lasiocampidae	*Artace sp.*	Bartholomew & Casey 1978	0.1286	0.0256
Lepidoptera	Lasiocampidae	*Odonestis pruni*	Niven & Scharlemann 2005'	0.2550	0.0473
Lepidoptera	Megalpygidae	*Megalpyge sp.*	Bartholomew & Casey 1978	0.6270	0.1922
Lepidoptera	Noctuidae	*Agrotis exclamationis*	Niven & Scharlemann 2005'	0.2000	0.0465
Lepidoptera	Noctuidae	*Agrotis pronuba*	Niven & Scharlemann 2005'	0.2733	0.6305

Lepidoptera	Noctuidae	*Cucullia lactucae*	Niven & Scharlemann 2005'	0.2850	0.0444
Lepidoptera	Noctuidae	*Plusia gamma*	Niven & Scharlemann 2005'	0.1200	0.0261
Lepidoptera	Notodontidae	*Apetaloides firmiana*	Bartholomew & Casey 1978	0.1690	0.0602
Lepidoptera	Nymphalidae	*Melitaea cinxia*	Niitepold & Hanski 2013	0.1000	0.0056
Lepidoptera	Nymphalidae	*Vanessa io*	Niven & Scharlemann 2005'	0.2044	0.0286
Lepidoptera	Nymphalidae	*Vanessa polychloros*	Niven & Scharlemann 2005'	0.2700	0.1052
Lepidoptera	Saturniidae	*Adeloneivaia boisduvalii*	Bartholomew & Casey 1978	0.9363	0.1091
Lepidoptera	Saturniidae	*Adeloneivaia subungulata*	Bartholomew & Casey 1978	0.4870	0.2092
Lepidoptera	Saturniidae	*Aglia tau*	Niven Scharlemann 2005'	0.1125	0.0443
Lepidoptera	Saturniidae	*Antheraea pernyi*	Niven & Scharlemann 2005'	0.8297	0.0495
Lepidoptera	Saturniidae	*Automerina auletes*	Bartholomew & Casey 1978	0.7200	0.3459
Lepidoptera	Saturniidae	*Automeris fieldi*	Bartholomew & Casey 1978	0.3940	0.0998
Lepidoptera	Saturniidae	*Automeris hamata*	Bartholomew & Casey 1978	0.5640	0.1687
Lepidoptera	Saturniidae	*Automeris jacunda*	Bartholomew & Casey 1978	0.5991	0.0795
Lepidoptera	Saturniidae	*Automeris zugana*	Bartholomew & Casey 1978	0.5523	0.0760
Lepidoptera	Saturniidae	*Dirphea agis*	Bartholomew & Casey 1978	0.1970	0.1892
Lepidoptera	Saturniidae	*Eacles imperialis*	Bartholomew & Casey 1978	1.1050	0.2742
Lepidoptera	Saturniidae	*Hyperchirica nausica*	Bartholomew & Casey 1978	0.2160	0.1053
Lepidoptera	Saturniidae	*Saturnia pavonia*	Niven & Scharlemann 2005'	0.1983	0.0951
Lepidoptera	Saturniidae	*Sphingicampa quadrilineata*	Bartholomew & Casey 1978	0.8180	0.2288
Lepidoptera	Saturniidae	*Syssphinx molina*	Bartholomew & Casey 1978	1.7570	0.3631
Lepidoptera	Sphingidae	*Deilephila elpenor*	Niven & Scharlemann 2005'	0.6500	0.2178
Lepidoptera	Sphingidae	*Enyo ocypete*	Bartholomew & Casey 1978	0.4145	0.2114
Lepidoptera	Sphingidae	*Erinnyis ello*	Bartholomew & Casey 1978	1.2100	0.3202
Lepidoptera	Sphingidae	*Hyles euphorbia*	Niven & Scharlemann 2005'	0.6500	0.2028
Lepidoptera	Sphingidae	*Madoryx oeclus*	Bartholomew & Casey 1978	1.6990	0.6652

Lepidoptera	Sphingidae	*Manduca corallina*	Bartholomew & Casey 1978	1.6183	0.0030
Lepidoptera	Sphingidae	*Manduca corallina*	Bartholomew & Casey 1978	1.6183	0.5906
Lepidoptera	Sphingidae	*Manduca lefeburei*	Bartholomew & Casey 1978	0.5710	0.2410
Lepidoptera	Sphingidae	*Manduca rustica*	Bartholomew & Casey 1978	2.8100	0.8009
Lepidoptera	Sphingidae	*Oryba achemenides*	Bartholomew & Casey 1978	2.8085	1.1960
Lepidoptera	Sphingidae	*Pachygonia drucei*	Bartholomew & Casey 1978	0.7020	0.3605
Lepidoptera	Sphingidae	*Pachylia ficus*	Bartholomew & Casey 1978	3.2250	1.0912
Lepidoptera	Sphingidae	*Perigonia lusca*	Bartholomew & Casey 1978	0.5583	0.2796
Lepidoptera	Sphingidae	*Protambulyx strigilis*	Bartholomew & Casey 1978	1.1097	0.1730
Lepidoptera	Sphingidae	*Xylophanes libya*	Bartholomew & Casey 1978	0.5590	0.2278
Lepidoptera	Sphingidae	*Xylophanes pluto*	Bartholomew & Casey 1978	0.8280	0.3739
Lepidoptera	Sphingidae	*Deilephila euphorbiae*	Niven & Scharlemann 2005'	0.3950	0.1397
Odonata	Aeshnidae	*Aeshna multicolor*	Henry & Harrison 2014'	0.6338	0.0460
Odonata	Aeshnidae	*Anax junius*	Henry & Harrison 2014'	1.2329	0.1335
Odonata	Libellulidae	*Libellula comanche*	Henry & Harrison 2014'	0.3882	0.1586
Odonata	Libellulidae	*Libellula luctuosa*	Henry & Harrison 2014'	0.2847	0.0459
Odonata	Libellulidae	*Libellula saturata*	Henry & Harrison 2014'	0.4311	0.1591
Odonata	Libellulidae	*Macrodiplax balteata*	Henry & Harrison 2014'	0.2189	0.0765
Odonata	Libellulidae	*Pachydiplax longipennis*	Henry & Harrison 2014'	0.1631	0.0626
Odonata	Libellulidae	*Pantala flavescens*	Henry & Harrison 2014'	0.1496	0.0624
Odonata	Libellulidae	*Pantala hymenaea*	Henry & Harrison 2014'	0.2997	0.3824
Odonata	Libellulidae	*Tramea lacerata*	Henry & Harrison 2014'	0.4387	0.0975
Odonata	Libellulidae	*Tramea onusta*	Henry & Harrison 2014'	0.3534	0.0868
Orthoptera	Acrididae	*Locusta migratoria*	Snellig *et al.* 2012	0.9630	0.1306
Orthoptera	Acrididae	*Schistocerca americana*	Rascon Harrison 2005	1.2200	0.1530
Orthoptera	Acrididae	*Schistocerca gregaria*	Niven & Scharlemann 2005'	1.7365	0.0043
Orthoptera	Acrididae	*Schistocerca gregaria*	Niven & Scharlemann 2005'	1.9600	0.1207

Table S3. Comparison of linear and breakpoint log-log models of flight metabolic rate across flying insects. Below 53 mg (theta= -0.63 in body mass in log-log form), flight metabolic rate scales hypermetrically while it scales hypometrically (not different from slope=0.67) above that mass. The breakpoint model has much higher support using Akaike's information criterion (AIC) than the standard linear model.

Model	Slope(s)	Std. err(s).	p (slope = 0)	p (slope = 0.67)	p (slope = 1)	Intercept	Theta	AIC	Akaike weight
Linear	0.98	0.04	<0.001*	NA	0.562	-0.62	NA	139.03	0.003
Break-point	Left: 1.15 Right: 0.49	0.07 0.17	<0.001* 0.004*	NA 0.305	0.027* 0.004*	-1.03	-0.63	127.34	0.997

Figure. 1 (A) Size comparison of biggest (*Melipona triplaridis* at 115 mg) and smallest (*Trigonisca buoyssoni* at 1 mg) stingless bees included in this study. (B) Metabolic rates of stingless bees with and without Q_{10} correction. (C) The thermal flight performance curve of *S. luteipennis* (n=30) indicates that flight metabolic rate for this stingless bee species is nearly independent of thorax temperature over a broad range, shown with a second order psolynomial fit. (D) Body segment temperature elevation above air temperature. Small bees (< 20 mg) had body temperatures 0.7-3°C above air temperatures, while large species (> 70 mg) had substantially elevated body segment temperatures. Lines show third order polynomial fits. (E) Wing beat frequency was constant across body size while load carriage abilities scaled isometrically (slope =1.05, Table S1). Total wing area scaled hypometrically, indicating that smaller stingless bees have proportionally larger wings. All multi species regression lines were plotted with PGLS. (F) Thorax and, abdomen mass scaled isometrically while head mass scaled hypometrically (slopes= 1.046 for thorax, slope=1.109 for abdomen and 0.7960 for head) with body mass across stingless bees.

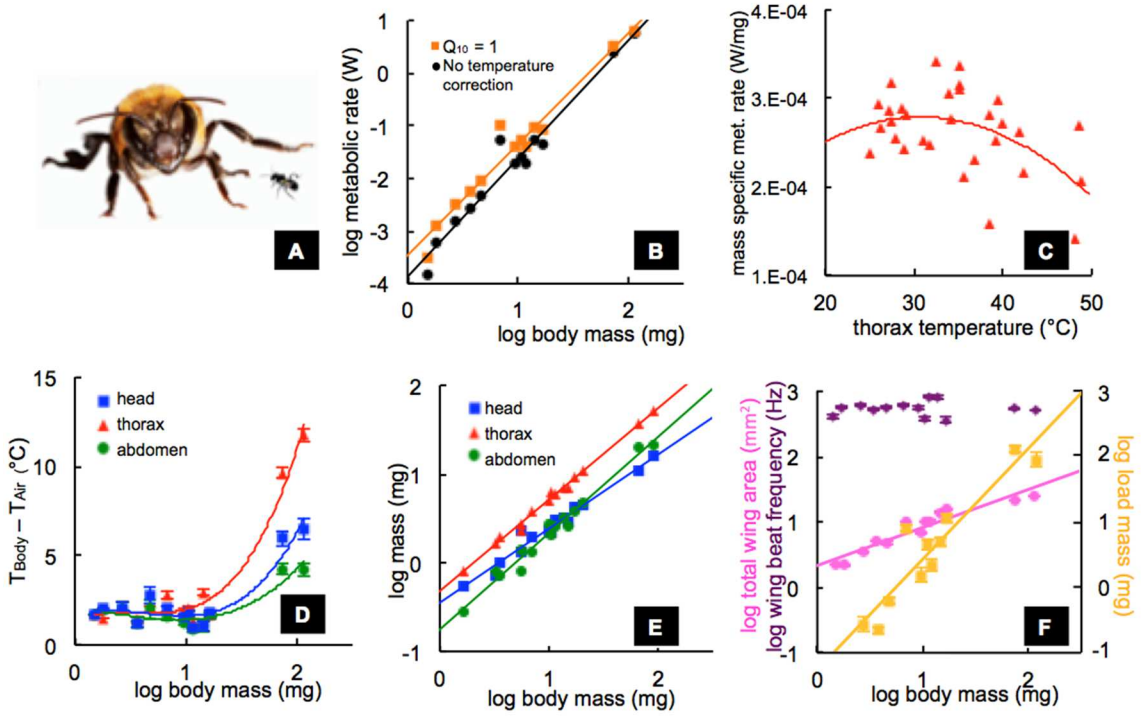


Figure 2. Flying while small costs less. Flight metabolic rate in insects below 53mg in body mass scales hypermetrically (slope =1.15) while flight metabolic rate in insect greater than 53mg scales hypometrically (slope=0.49, not significantly different from 0.67; Table 3).

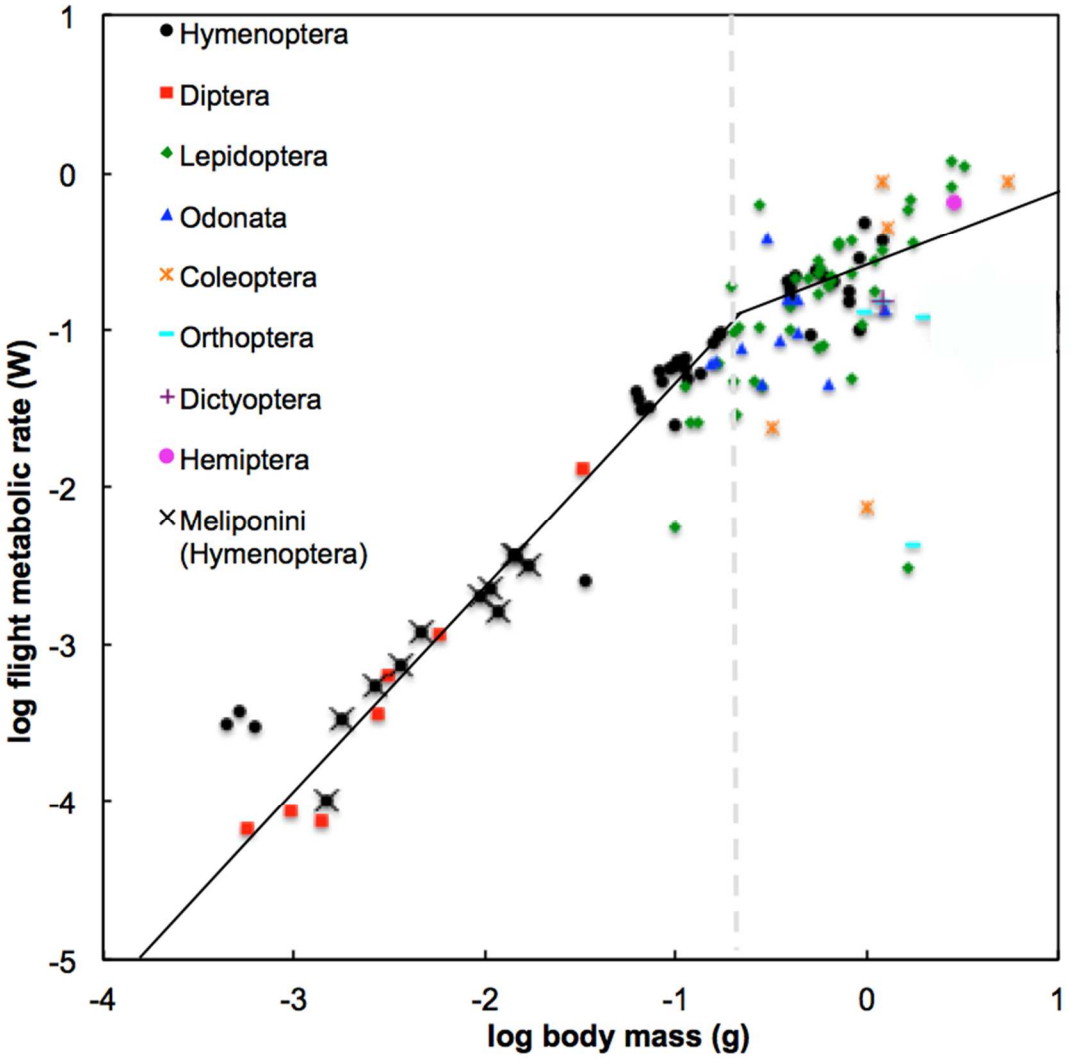


Figure S1. Phylogenetic tree of stingless bee species included in this study based on relationships found in Rasmussen & Cameron 2007 and 2009. All branch lengths are set equal to one because of the absence of some species from available molecular phylogenies of Meliponines. Phylogenetic independent contrasts demonstrate that phylogeny is not a significant factor in our analysis (Table 1). *Plebeia spp. (frontalis)* is undescribed at this time. PGLS analysis was done with and without this species included and did not yield different results. Average body mass ±SE are indicated next to species names and miniaturized lineages are specified with an asterisk according to Michener (2001) and Camargo (2013).

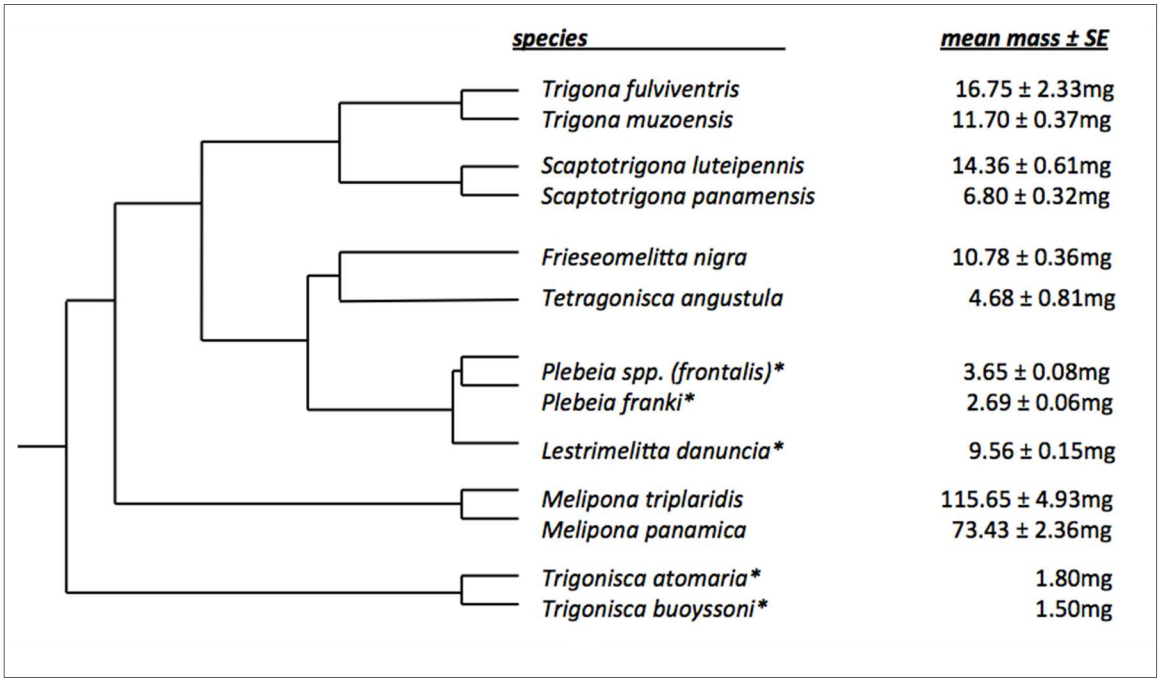


Figure S2. (A) Scaling relationships of forewing (blue) and hindwing (red) length (squares), and widths (circles). All scaled hypometrically with body mass (Table S1). (B) Scaling of total wing area (black), forewing area (blue), and hindwing area (red) with body mass. Forewings are proportionally larger in smaller bees than hindwings. All scaling parameters are listed in Table S1.

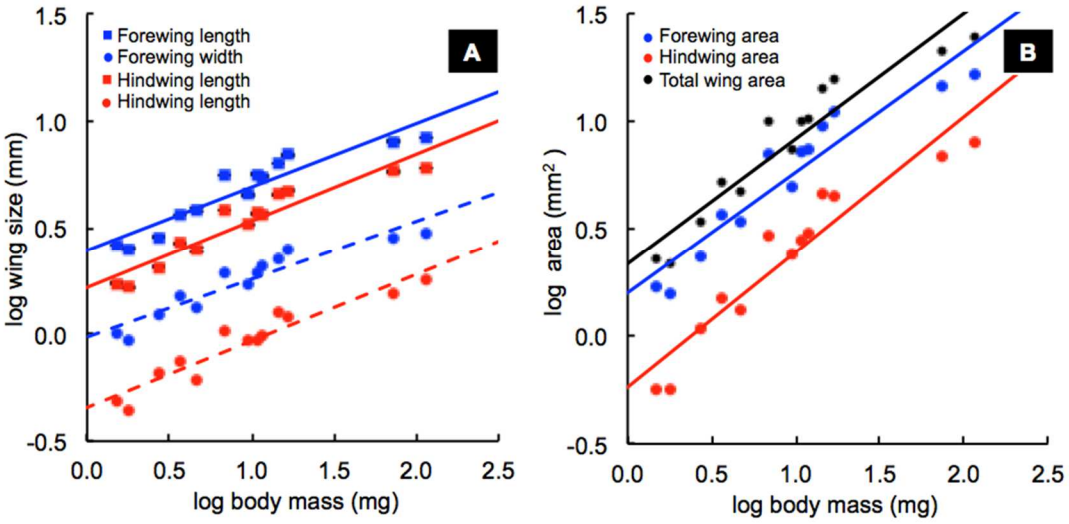
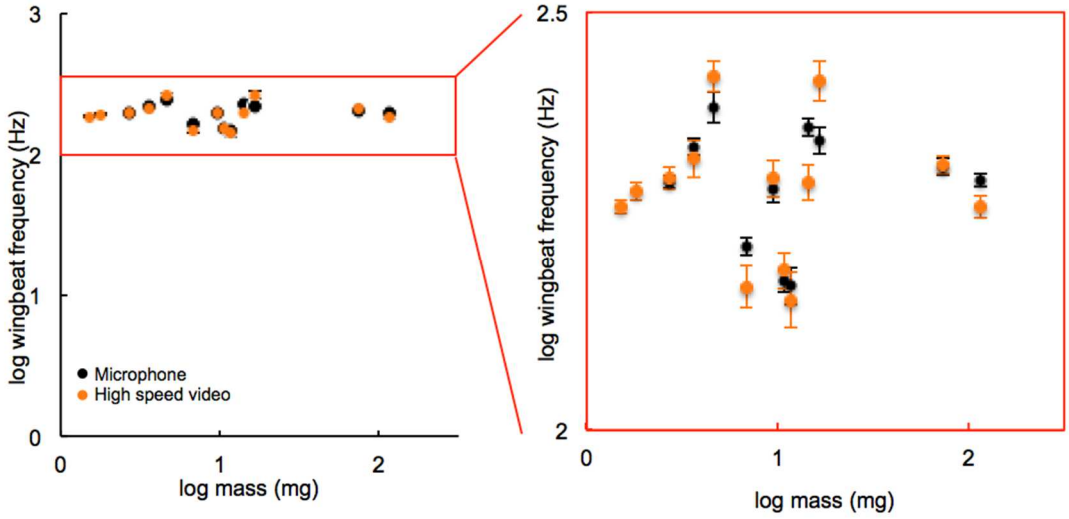


Figure S3. Comparison of microphone and high speed video methods of wing beat frequency measurement. There was no significant relationship between mass and wing beat frequency among all species (slope= 0.02, Adj. R^2=0.066,*P*=0.524). Average wing beat frequency across species = 204.6 ± 8.3 SE beats/sec. Each point represents the average wing beat frequency within a species ±SE.



REFERENCES

Main Text References:

1. J. Kingsolver, R.B. Huey. *Evol. Ecol. Research* **10**, 251-268. (2008).

2. W.U. Blanckenhorn. *Q. Rev. Biol.* **75**, 385-407. (2000).

3. J. Hanken, D.B. Wake. *Ann. Rev. Ecol. Syst*. **24**, 501–519 (1993).

4. W. Eberhard, W.Wcislo. *Adv. Insect Physiol.* **40**, 155-213 (2011).

5. J.H. Brown *et al., Metabolic Ecology: A Scaling Approach* (Wiley-Blackwell. (2012).

6. R. Dudley. *The Biomechanics of Insect Flight: Form, Function, Evolution.* (Princeton University Press, Princeton. (2002).

7. R.K. Suarez. *Physiol. Biochem,* **73**, 765-771. (2000).

8. B. Sacktor. Biochem. Soc. Symp. **41**,111-131. (1976).

9. G.A. Bartholomew, T.M.Casey. *J. Exp.Biol.* **76**, 11-25. (1978).

10. T.M. Casey *et al., J. Exp.Biol.* **116**, 271-289. (1985).

11. C.A. Darveau, *et al., J. Exp.Biol.* **208**, 3593-3602. (2005).

12. J.E. Niven, J.P.W. Scharleman. *Biol. Lett.* **1**, 346-349. (2005).

13. C. Rasmussen, S.A. Cameron. *Biol. J. Linn. Soc.* **99**, 206–232 (2010).

14. J. Felsenstein, *Am. Nat.* **125**, 1–15 (1985).

15. P.H. Harvey, M.D. Pagel. *The Comparative Method in Evolutionary Biology.* Oxford University Press, New York, NY. (1991).

16. J.M.F Camargo in *Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region*, J.S. Moure, G.A.R Urban de Melo (Sociedade Brasileira de Entomologia; Curitiba, Brasil. (2007).

17. J.M.F. Camargo in *Pot Honey: A Legacy of Stingless Bees,* P Vit, S.R.M. Pedro, D.W. Roubik, Ed. (Springer Science + Business, New York, 2013) pp 19-34.

18. C.D. Michener. *J. Kansas Entomol. Soc.* **74**, 231-236 (2001).

19. M.E. Dillon, R. Dudley. *J. Exp. Biol.* **207,** 417-425. (2004).

20. G.A. Bartholomew, T.M.Casey. *J. Therm. Biol* **2**:, 173-176. (1977).

21. B. J. Heinrich. *The Hot-blooded Insects: Strategies and Mechanisms of Thermoregulation* (Harvard University Press, Cambridge, MA, 1993).

22. May ML. *J. Comp. Physiol. B* **144**, 229–40 (1981).

23. D.M. Unwin, S.A. Corbet. *Physiol. Entomol.* **9**,115– 21 (1984).

24. D.W. Roubik, F.J.A. Peralta, *Acta Amazonica* **13,** 453-466 (1983).

25. B.J. Heinrich. *Exp.Biol.* **54**, 141-147 (1971).

26. J.F. Harrison *et al., J. Exp.Biol.* **4**,805-814. (2001).

27. S.P. Roberts, J.F. Harrison. *Am. Zool.* **38**, 492-502. (1998).

28. J.F. Harrison *et al., Science* **274**, 88-90. (1996).

29. S.P. Roberts *et al., J. Exp.Biol.* **15,**2321-2331. (1998).

30. D.N. Byrne. *J. Exp. Biol.* **135,** 9-23 (1988).

31. J.F. RSPaH . *J. Exp.Biol.***202**,1523-1533. (1999).

32. M.L. May. *J. Exp.Biol.***198**,2385-2392. (1995).

33. T.M. Casey. *Insect Flight*, eds Goldsworthy GJ & Wheeler CH (CRC, Boca Raton), pp 257-272. (1989).

34. T.M. Casey. *J. Exp.Biol.***88**,133-145. (1980).

35. T.M. Casey. *Physiological Zoology* **54**,362-371. (1981).

36. B.J. Heinrich, T.M. Casey. *J Comp Physiol B* **82**,195-206. (1973).

37. B. Heinrich. *J. comp. Physiol.* **96**,155-166. (1975).

38. F.O. Lehmann. *J Comp Physiol B* **169**,165-171. (1999).

39. W.J. Yurkiewicz, T. Smyth Jr. *J. Insect Physiol.* **12**,189-194. (1966).

Supplementary References

1. Priyadarshana W.J.R.M. R Verision 1.2 (2016).
2. J. Henry, J.F. Harrison *J. Exp. Biol.* **217,** 3447-3456 (2014).
3. E.P. Snelling *et al., J. Exp. Biol.* **215,** 3317-3323 (2012).
4. B. Rascon & J.F. Harrison. *J. Insect Physiol.* **51,** 1193-1199' J.F.Harrison. (2005).
5. Bartholomew, Barnhart. *J. Exp. Biol.* **111,** 131-144 (1984).
6. Josephson *et al., J. Exp. Biol.* **204,** 4125-4139 (2001).
7. Chappell. *Phys.Zool.* **57,** 581-589. (1984).
8. Auerswald *et al., J. Exp. Biol.* **201,** 1651-1657 (1998).
9. C.J. Klok et al. in prep.
10. Heymann, Lehmann *J. Exp. Biol.* **209,** 1662-1677' (2006).
11. Lehmann, Schutzner *J. Insect Physiol.* **56,** 543-550 (2009).
12. Lehmann. *Science* **294,** 1926-1929 (2001).
13. Niitepold, Hanski. *J. Exp. Biol.* **216,** 1388-1397' (2013).
14. J.F. Harrison *et al., Physiol. Biochem. Zool.* **78,** 153-162' (2005).
15. Darveau *et al., J. Exp. Biol.* **217,** 536-544 (2014).
16. Hedenstrom *et al., Func. Ecol.* **15,** 417-422 (2001).
17. Bennett *et al., J. Econ. Entomol.* **106,** 1089-1097 (2014).
18. Chai *et al., Physiol. Biochem. Zool.* **72,** 145-155 (1999).
19. Clark, Dudley *Physiol. Biochem. Zool.* **83,** 654-662'. (2010).
20. K. Welch *et al., J. Exp. Biol.* **210,** 2146-2153. (2007).

21. VonBusse *et al., J. Exp. Biol.* **216,** 2073-2080 (2013).

22. Voigt *et al., Biol.Lett.* **7,** 793-795. (2011).

23. Voigt Winter *J.Comp.Physiol. B.* (1999).

24. Suarez *et al., Comp. Biochem. Physiol. A* **153,** 136-140' (2009).

25. Voigt *et al., J. Exp. Biol.* **215,** 4340-4344 (2012).

26. Voigt *J. Exp. Biol.* **216,** 1516-1521 (2013).

27. Garamszegi. L.Z. Springer-Verlag Heidelburg, Berlin (2014).

CHAPTER 4

DOES BODY SIZE DICTATE SUSCEPTIBILITY TO RISING TEMPERATURES? THE SCALING OF FLIGHT UPPER THERMAL TOLERANCE AMONG STINGLESS BEES

ABSTRACT

Climate change has caused global temperatures to rise, a trend that will continue into the foreseeable future. This will impact pollinators around the globe. Tropical regions that contain the vast majority of our world's biodiversity are most endangered by warming trends. It is vital that we understand the physiology of pollinators that provide invaluable pollination services throughout the tropics, ensuring continued biodiversity and food supply. Insect pollinators, particularly bees, are dependent on air temperature to regulate physiological and behavioral processes as ectotherms. Thus, their performance as pollinators will be negatively impacted if they are unable to cope with a warmer climate. It is unknown whether smaller or larger ectotherms will fare better in the predicted temperature changes throughout the tropics. Smaller ectotherms generally conform more closely to air temperature while larger insects may produce a greater volume of heat, allowing them to be active in cooler temperatures but limiting their time spent in hotter conditions. Thus, smaller ectotherms should perform better than larger ectotherms in hotter temperatures based on current knowledge. Here I measure flight performance across a range of air and body temperatures using ten species of stingless bees that vary in body size from 2-120 mg in body mass. I measured leaf and flower surface temperatures and air temperatures in sun and shade in the tropical forest canopy, where

stingless bees are found foraging, using a canopy crane. At the same time, I caught bees and measured thorax temperatures relative to air temperature to document the range of temperatures experienced by bees during flight in field conditions. Smaller species flew with body temperatures much closer to air temperature than larger species, which fly at temperatures up to 10°C in excess of air temperature. This is partially explained by the scaling of heat gain and loss as a function of body volume; smaller ectotherms gain and lose heat more rapidly. I also caught foraging bees of each species and flew them in a lab setting while controlling air temperature and measuring metabolic rate. The shape of thermal performance curves varied by species. The critical thermal maximum temperature at which flight ceased was lower in smaller species than large. This indicates that, despite conforming to air temperature during flight, smaller ectotherms do not necessarily tolerate higher temperatures. Larger ectotherms, especially those that fly or actively thermoregulate, might tolerate higher temperatures because they produce more heat through metabolic activity.

INTRODUCTION

Rising and fluctuating global temperatures associated with human-induced climate change will have a significant impact on all organisms, but ectotherms (organisms with bodily functions dependent on ambient temperature) will likely be the most strongly affected by these changes (Angilletta 2006, Bozinovic et al. 2011). Insect pollinators are included in this group. They depend on ambient temperature to reach operational body temperature (Kingsolver & Huey 2011). Temperatures that are too high require them to behaviorally thermoregulate by seeking shelter from solar radiation or to neglect food foraging trips in order to find water sources to cool their bodies (Schmaranzer 2000). High ambient temperatures also cause insects to have higher metabolic rates regardless of activity state. The rising temperatures and frequent temperature fluctuations associated with climate change will affect the flight performance of pollinators (Kingsolver & Huey 2008, Kremen et al. 2004, Klein et al. 2007). Pollinators, such as bees, that must forage to support a colony might not be able to sustain the energetic demands of higher body temperatures. To date there are few studies exploring how ecologically significant species such as pollinators may respond to warmer temperatures physiologically (Tsuji et al. 1986, Parmesan et al. 1999, Crozier & Dwyer 2006) but is imperative that we understand thermal sensitivity among ectotherms to predict how they might fare in the future (Dillon et al. 2010, 2016, Kingsolver & Woods 2016).

Most bees thermoregulate behaviorally by sun- and shade-seeking to either warm or cool themselves. They can also use evaporative cooling through behaviors like tongue-

lashing in which they spit out water or nectar and let it evaporate from the cuticle, and through defecation. Some bees also forage for water to take back to their nests when it is hot in order to evaporatively cool the nest (Stone 1994, Woods et al 2005, Roberts & Harrison 1998, Roberts et al. 1998). Bumblebees, carpenter bees, and honeybees can endothermically warm themselves by shivering their flight muscles when it is too cold and shunt heat via hemolymph to different sections of the body (Heinrich 1972, 1974, Chappell 1982) but there is no known equivalent for when conditions are too hot.

In general, body size plays an important role in how insects deal with changing ambient temperatures (Peters 1983, Darveau et al 2002, Hulbert & Else 2000, West 2002) and temperature affects the cost of living in all ectotherms (Darveau et al.2002, Hulbert & Else 2000). Large species get hotter faster in flight because their flight muscles produce a great deal of metabolic heat. Shivering, for example, is size-dependent; only larger bees are known to use shivering as a warming mechanism (Roberts & Harrison 1998). The rate at which bees and other insects) gain and shed heat is also size dependent. Smaller species conform closely to air temperature due to low surface area to volume ratios while larger bees are often hotter than air temperature (up to 15°C), especially during flight (Stone & Willmer 1989). This may limit the amount of time that bees have for activity. Larger bees may be limited by getting too hot in the afternoon hours while smaller bees may be limited by temperatures that are too cool for flight in the morning and evening (Stone 1994, Lehmann 1999). Other thermoregulatory behaviors may also be size dependent and have an impact upon the activity patterns and performance of bees.

Flight is the primary mode of transportation that allows bees to pollinate millions of wild and cultivated plant varieties. Flight is a very energetically costly behavior that causes metabolic rates to rise upwards of thirty times higher than resting in some ectotherms (Darveau et al. 2002, Reinhold 1999). Flight performance is size-dependent; smaller species generally have higher wing beat frequencies and higher mass-specific metabolic rates than larger species among all insects (measured mostly in larger insects), though I have previously shown hypermetrics scaling of flight metabolic rate among smaller stingless bees (Duell, Chapter 1) . In hotter conditions, individual bees may see higher energetic costs for flying to collect pollen and nectar (Lehmann 1999). Workers may die faster because of difficulty thermoregulating, which can cause the collapse of colonies (Norgate et al. 2010) if bees are unable to acclimate and adapt quickly to warmer air temperatures. The community of pollinators may be disrupted (Kingsolver and Huey 2008), leading to decline in plant diversity and human food supply. Therefore, it is imperative that we understand how thermal sensitivity scales with body mass across pollinators, so that we might protect them and the ecosystem services they offer.

Stingless bees are an ideal group for studying the scaling of thermal sensitivity because species vary in size by three orders of magnitude in body mass (Michener 2001) and have a fairly well resolved phylogeny (Rasmussen & Cameron 2011, Camargo 2013). They are common throughout the world's tropical regions and serve as generalists that pollinate many crops and wild flowering plants (Vit et al. 2013, Roubik 1989, 2000). In total, over 500 species have been identified (Michener 2001). A single species can forage at 100 species of plant in a year's time, which contributes to the preservation of

biodiversity and food security in the tropics (Roubik 1989, 2000, Vit et al. 2013). Stingless bees are the most numerous pollinators in many regions of South America, Central America, Africa, and Asia. Because of their distribution in the tropics, they may be disproportionately affected by climate warming.

METHODS

Collection: Forager stingless bees were identified and collected while foraging at flowers on the ground and in the forest canopy, at honey-water feeders, or returning to nest sites. Species collected included *Melipona panamica, Scaptotrigona luteipenis, Trigona muzoensis, Trigona fulviventris, Tetragonisca angustula, Plebeia franki*, *Plebeia frontalis,* and *Trigonisca atomaria* (field measurements only). These species vary in body mass from 1-120 mg among foragers. All analyses were performed using generalized least squares regression (PGLS) in order to account for phylogenetic signal (Revell 2010, Garamszegi 2014, Harvey & Pagel 1991, Felsenstein 1985). A tree was adapted from Rasmussen & Cameron (2012). Locations for collection included Gamboa near Parque Nacional Soberanía, Barro Colorado Island, Parque Nacional Metropolitano in Panama City, Curundú, Santa Rita Arriba, and Fort San Lorenzo. Bees were brought back to Smithsonian Tropical Research Institute laboratory facilities in Gamboa, Panamá in falcon tubes and supplied with 50% sucrose on a cotton ball during transportation.

Field temperature measurements: The thorax temperatures of bees were measured using a grab and stab technique (Stone & Willmer 1989) in which bees were caught flying in a transparent Ziploc bag and stabbed through the bag with a micro thermocouple. Hands were heavily gloved and the Ziploc bag was placed on a thick piece

of foam insulation to avoid heat transfer. Thorax temperatures were read on a BAT-12 thermocouple meter within two seconds. Air temperature was recorded at the same time as thorax temperature. These measurements were taken using the Smithsonian Tropical Research Institute Canopy crane at near Fort San Lorenzo in the Republic of Panamá. These data represent collection on five separate days between June and September of 2016. Measurements were taken between 9-pm and 3pm based on crane availability, which does not reflect the full amount of time bees might spend foraging throughout the day in the canopy. It is likely much greater thermal variation in the canopy sampled than is reflected by these data due to seasonal and weather variation, but the data reflect conditions during which bees were caught and measured at the canopy crane site.

Commonly used surfaces such as leaves and flowers were also measured using the micro thermocouple from the canopy crane in Fort San Lorenzo in the sun and shade. To do this, the thermocouple was placed on top surfaces of leaves and flowers in the sun and shade. These measurements were used to assess the thermal conditions bees usually encounter along with air temperatures, and the experienced thorax temperatures. The differences in temperature among leaf and flower surfaces and air were assessed with two-factor ANOVA. When sun vs. shade and the surface were found to be significant, an additional one-factor ANOVA was performed with post-hoc Tukey-Kramer comparison tests among the temperatures of sunny leaves, shady leaves, sunny flowers, shady flowers, and air. Additional measurements of average annual high temperatures and record high temperatures from 1996-2016 were gathered from Smithsonian Tropical Research Institute climatological databases. These measurements were taken by

instruments mounted on the canopy crane at tree crown level where leaf, flower, air, and bee temperatures were also measured (Physical Monitoring Program of the Smithsonian Tropical Research Institute). They were used to compare critical thermal maxima with recorded air temperature data and forecast the effects of climate warming on bees.

Flight metabolic rates: In order to determine how flight metabolic rates varied across air temperatures, I designed a thermal chamber that consisted of a 14 gallon Rubbermaid storage container and strips of thin plastic sheeting taped across the open front to keep the heat inside the thermal chamber. Two 100 watt flood light bulbs were connected to an Inkbird Heating Cooling Thermostat temperature controller that monitored the temperature in the flight chamber (the glass chamber used for respirometry measurements) and the Rubbermaid thermal chamber. The temperature controller turned the 100W light bulbs on or off to regulate the temperature within 0.1°C of the desired set temperature. The chambers took 2-5 minutes to warm depending on the initial and desired air temperatures. Temperatures used for respirometry ranged from 25-45°C. A third LED light was also attached between the heat bubs to stimulate bees to fly without shedding heat extra heat into the chamber (Menzel & Greggers 1985). A small fan was setup in the back of the chamber to circulate air and ensure thermal homogeneity throughout the thermal chamber.

A flow-through respirometry system consisting of an air pump (air flow maximum 2L/min), an OMEGA flow-meter (range of 10-200ml/min) to regulate air flow for bees of different sizes, a glass Fluon^{TM}-lined flight chamber, and a LiCor 6252 CO_2 analyzer. Data were collected using Expedata version 7.2. Incurrent air was scrubbed of

water vapor using a scrubber column of drierite placed between the air pump and flow meter. A separate scrubber column consisting of ascarite and soda lime was included to remove CO_2 from the incurrent air. The CO_2 analyzer was calibrated daily using a calibrated CO_2 gas cylinder of 1221 ppm CO_2. I used four different cylindrical glass flight chambers with volumes of 15ml, 70ml, 150ml, and 550ml and chose the smallest chamber that bees of each species would fly in. Flow rates were adjusted to chamber size so that the 95% washout time for that chamber was approximately 45 sec; flow rates ranged from 150 ml min^{-1} in the smaller chambers with smaller bees to 1000 ml min^{-1} inthe largest chamber and bees (see Chapter 1 for more information).

Individual bees were placed in the respirometry chamber and stimulated to fly by gentle shaking and movement of the chamber using heavily gloved hands to avoid heat transfer or insulation of the chamber. Bees were stimulated to fly until they could no longer fly at the temperature they were tested for. When a bee stopped flying, it was removed from the chamber, placed in a Ziploc bag on foam insulation, and its body temperature was immediately measured. It was kept inside the Rubbermaid thermal chamber to avoid cooling from the laboratory air temperature. Each bee was stabbed in the thorax with a micro thermocouple within two seconds of removal from the flight chamber. The temperature was read from a BAT-12 thermocouple meter. The set air temperature and actual air temperature were also recorded at this time. Immediately afterward, each bee was weighed.

For each species, 30-50 bees were used to build thermal performance curves of mass-specific flight metabolic rate vs. temperature. Thorax and air temperature were

compared in each species to show whether bees of each species conformed to air temperature in flight. Each bee experienced a single set temperature. These data were analyzed using linear and non-linear model comparisons. All possible biologically relevant models were compared to determine the best possible fit to flight metabolic rate data. The likelihood of models was compared using Akaike's information criterion (AIC) and the conformation of thorax temperature to air temperature was tested similarly by comparing the models for thorax and flight metabolic rate vs. temperature and flight metabolic rate vs. thorax temperature (Table 2).

The critical thermal maximum for flight was the temperature at which bees could no longer fly (Lutterschmidt & Hutchison 1997). Bees could not be stimulated to fly above this temperature and often lacked coordinated movements. This was determined by flying many bees at different temperatures and finding the minimum temperature at which they would not fly and lost coordination during the flight metabolic rate measurments discussed above. Q_{10} was calculated for each species by comparing the metabolic rates of bees flying at air temperatures separated by 10°C between 25-35°C.

RESULTS

In the field, larger bees had a greater elevation of thorax over air temperature (slope = 0.759, $p = 0.008$, std. err = 0.177, $\lambda = 0.000$). Smaller species conformed very closely to air temperature, but even large species were only a few degrees above air temperatures. This difference may be greater in cooler air conditions when bees might actively warm themselves. Leaf and flower surfaces were always hotter in the sun, and leaves were

always warmer than flowers. Air temperatures were coolest according to two-factor ANOVA followed by post-hoc Tukey-Kramer comparisons (Fig 1. Table 3).

Thermal performance curves varied greatly by species; Flight metabolic rate increased linearly with increasing temperature in *P.frontalis* (r^2= 0.17, p=0.02, AIC= -514.7, Akaike weight= 0.42, quadratic r^2= 0.24, =0.20, AIC=-515.3, Akaike weight= 0.58 [Fig. 2, Table 3]). The relationship between flight metabolic rate and air temperature followed a quadratic function in *T. fulviventris*. It was unclear whether this relationship followed a negative linear function or quadratic function in *S. luteipenis* based on model comparisons with AIC (linear model p=0.02 [Fig. 2, Table 3]). In all other species, there was no significant linear or nonlinear relationship between flight metabolic rate and air temperature (Fig. 2, Table 3).

Q_{10} did not scale with body mass (slope =0.017, p=0.820, std. err = 0.069) and was not dependent on phylogeny (λ=0.00) using PGLS (Fig. 3, Table 3). The scaling of thorax-air temperature was isometric (slope = 0.98 (log-log), p=0.001, λ= 0.00) in the lab. As in the field, smaller species conformed more closely to air temperature during flight. Larger species were up to 10°C hotter than air temperature. Critical thermal maximum for flight scaled hypermetrically, though not significantly different from isometry (slope =1.5 in log-log form, p=0.114). This was also not dependent on phylogeny (λ=0.00, Fig. 3, Table 3). Larger species had higher critical thermal maxima than smaller species. When compared with typical thorax temperatures experienced in the field, the critical thermal maximum was between 3-10°C hotter. The critical thermal maxima of some species,

especially smaller ones, falls within the recorded average annual high temperatures and record high temperatures (Fig 5).

DISCUSSION

In the field, bees have a range of microclimates to choose from for behavioral thermoregulation (Dillon et al. 2012, Potter et al. 2013, Woods et al 2015). They may seek shade when the air is too hot in the sun or seek sunny surfaces to warm up when conditions are cool. I found that leaf surfaces were hottest, followed by flowers and air. These differences may be light, weather, and seasonally dependent. All surfaces are warmer in the sun than in the shade in the canopy (Fig 2, Table 1). Others (Dillon et al. 2012, Woods et al 2015) have found similar microclimate differences in tropical and temperate forests and record diverse microclimate use by bees (Dillon et al. 2012, Potter et al. 2013, Woods et al 2015).

Flight metabolic rate did not differ across the range of air temperatures (25-45°C) tested in most species examined. This suggests that flight metabolic rate is maintained across temperatures until conditions get too hot and bees are no longer able to fly, hitting their critical thermal maximum for flight behavior. There were few exceptions. Others have found variation in flight metabolic rate with air temperature as well. Honeybees may increase, decrease or maintain flight metabolic rate over a range of temrpeatrues depending on loading, caste, and season (Heinrich 1980, Roberts & Harrison 1999, Harrison et al. 2001). Flight metabolic rate increased linearly with increasing air temperature in *P. frontalis* until the critical thermal maximum where they stopped flying.

No decrease in metabolic rate was observed at this point. This suggests that *P. frontalis* will pay a higher metabolic cost to fly as the climate warms and encounter its critical thermal maximum more often, limiting the amount of time spent on activities such as foraging. The relationship between flight metabolic rate and temperature followed a quadratic function in *T. fulviventris* with a peak metabolic rate at 39°C in thorax temperature. Metabolic rate decreased strongly from this peak to the critical thermal maximum in this species. This demonstrates a fairly narrow range of temperatures at which *T. fulviventris* performs maximally, possibly limiting its daily time spent foraging, especially during hotter and colder seasons. It was unclear whether a linear nonlinear model better fit the relationship of flight metabolic rate and temperature in *S. luteipenis* as both were somewhat supported by AIC (Table 2). Regardless, metabolic rate also decreased before hitting the critical thermal maximum in this species. Based on thes variation in thermal performance curves, there no differences in flight performance trends across temperatures based on body size. This has been hinted, but not tested, by examining whether worker bees of different sizes or more or less likely to fly at different temperatures, with no effects (Couvillon 2010).

The critical thermal maximum for flight scaled hypermetrically with body mass. This demonstrates that smaller species had lower critical thermal maxima than larger species (Table 3) and refutes some aspects of the temperature-size rule (Kingsolver & Huey 2008, Dillon & Frasier 2013, Walczynska et al. 2016, Oyen et al. 2016). The temperature -size rule (Kingolver & Huey 2008, Walczynska et al 2016) implies that smaller ectotherms are better suited to warmer climates based on data showing that

smaller ectotherms are more numerous closer to the equator and that they conform more closely to air temperature than larger species (Casey 1992, Edeline et al. 2013, Huey & Kingsolver 1989). However, lower critical thermal maxima in smaller stingless bee species suggests that, though they do conform to air temperature more closely than larger species, they do not tolerate higher temperatures as well. I find no evidence that smaller species perform better in flight in the heat, or prefer higher temperatures. Larger stingless bee species appear to be better suited to higher temperatures than smaller species. Perhaps this is due to more frequent high temperature exposure. Larger bees produce much more metabolic heat that warms their bodies up to 10°C warmer than the surrounding air; therefore the temperatures they directly experience during daily foraging are likely higher than those experienced regularly by smaller stingless bee species. Measurements in this study were taken on fairly average summer days June-August, but these differences may be even more severe during the hottest (when larger bees shed excess heat from metabolic production) and coolest days (when larger bees may intentionally warm themselves by shivering) of the year.

All stingless bee species flew fly at temperatures 3-10°C lower than their critical thermal maximum in the field (Fig 4). However, some species have critical thermal maxima within the range of the average annual high temperatures and record high temperatures from 1996-2016. Even in the climate scenario that average temperatures will only rise 2°C warming over the next fifty years (Schleussner et al 2016), stingless bees will be flying in hotter air temperatures. As air temperatures approach the CT_{max} of some species, they will be forced to fly less often or risk heat injury. This effect will be

much more pronounced if the climate exceeds a 2°C increase in temperature. The high humidity found in tropical regions where stingless bees are common will likely make exacerbate heat stress, as evaporative cooling is less effective (Mellanby 1932).

Smaller species with lower critical thermal maxima are at greater risk and possibly are currently unable to forage during midday hours during the hottest days of year. Increases in frequency or intensity of these hottest days will further restrict foraging. To deal with these issues, smaller stingless bee species may seek shade and avoid hot leaf surfaces (Dillon et al. 2012, Potter et al. 2013, Woods et al 2015). They may also use evaporative cooling, though this has not been documented among stingless bees.

Decreased time for foraging due to thermally stressful or lethal temperatures likely has an indirect effect on stingless bee fitness by limiting food supply to the queen and/or lessening the number of workers available to perform non-foraging tasks. When queens die, colonies are at greater risk of collapse and the effective population size may become limited, therefore limiting genetic and phenotypic diversity among populations (Newman & Pilson 1997). If queens are directly exposed to high temperatures within colonies, there may be selection for higher thermal tolerance over time through selection on a myriad of traits that are responsive to temperature. Stingless bees choose nest cavities base don how easily they can be thermoregulated, at least partially, which directly impacts queen and brood exposure to stressful temperatures (Jones & Oldroyd 2007). Brood of *Scaptotrigona depilis* are especially susceptible to high and low temperature extremes and workers use social thermoregulation to maintain temperatures

as close to optimal for brood as possible (Vollet-Neto et al. 2015). There is no data available on stingless bee queen thermal tolerance. However, this does not mean the same selection process or results will occur in non-reproductive foragers. Further, the frequency of stressful high temperatures and rate of temperature change may influence the strength of selection, traits selected upon, and heritability of thermal tolerance in future generations of stingless bees, as found in fruit flies (Chown et al. 2009, reviewed in Chown et al. 2010 and Angiletta 2009). A greater understanding of thermal tolerance heritability within this clade is essential to determining how they will fare in the future of climate change (Huey et al. 2010, Terblanche et al. 2011).

Future work should investigate why thermal performance curves vary by species, why critical thermal maxima scale with body size among stingless bees, and the evolutionary consequences of stressful high temperature exposure. The evolutionary consequences of higher air temperatures on stingless bee size and species survival will depend on the their abilities to behaviorally thermoregulate as well as their metabolic performance. These results indicate that thermal sensitivity variation with body size may have large evolutionary consequences moving forward into hotter times.

TABLES & FIGURES

Table 1. A) Two-Factor ANOVA and B) post-hoc Tukey-Kramer comparisons of leaf, flower, and air temperatures in the sun and shade. $T_{avg}(1)$and $T_{avg}(2)$ represent the mean temperature for the first and second terms of each comparison.

Factor	Df	MS	F	P
Sun/shade	2, 495	31.94	44.09	<0.001
Leaf/flower	1, 495	22.99	31.74	<0.001
Interaction	1, 495	0.38	0.52	0.47
Comparison	$T_{avg}(1)$	$T_{avg}(2)$	Diff	p
Sun-Shade	27.17	26.80	0.37	<0.001
Sun-Air	27.17	26.19	0.98	<0.001
Shade-Air	26.80	26.19	0.61	<0.001
Leaf-Flower	27.23	26.75	0.48	<0.001
Leaf-Air	27.23	26.19	1.03	<0.001
Flower-Air	26.75	26.19	0.55	<0.001

Table 2. A) Thermal performance curve parameters for all species comparing linear and quadratic models, which had the highest likelihoods among all possible models using AICs. Significant fits with the highest support are bolded. B) Comparison of significant fight metabolic rate vs. temperature models.

A Species	Model	Temp. variable	Estimates	P	AIC	Akaike weight	R^2
Melipona panamica	Linear	Thorax	a = -0.002716 b = 0.000116	0.39 0.08	-360.6	0.72	0.09
	Quadratic	Thorax	a = -0.000006 b = 0.000551 c = -0.010553	0.76 0.70 0.70	-358.7	0.28	0.09
	Linear	Air	a = 0.002422 b = -0.000004	0.17 0.95	-357.3	0.72	<0.01
	Quadratic	Air	a = -0.000002 b = 0.000126 c = 0.000372	0.86 0.86 0.98	-355.4	0.28	<0.01
Plebeia franki	Linear	Thorax	a = 0.000193 b = 0.000002	<0.01 * 0.26	-499.5	0.67	0.05
	Quadratic	Thorax	a = <0.000001 b = -0.000021 c = 0.000567	0.48 0.52 0.29	-498.0	0.33	0.06
	Linear	Air	a = 0.000159 b = 0.000003	0.04* 0.15	-500.4	0.58	0.07
	Quadratic	Air	a = 0.000001 b = -0.000034 c = 0.000766	0.27 0.31 0.17	-499.8	0.42	0.12
Plebeia frontalis	**Linear**	**Thorax**	**a = -0.000714 b = 0.000033**	**0.13 0.02***	**-167.6**	**0.72**	**0.43**
	Quadratic	Thorax	a = 0.000001	0.74	-165.7	0.28	0.44

			b = -0.000045 c = 0.000595	0.85 0.88			
	Linear	Air	a = -0.000645 b = 0.000031	0.18 0.03*	-166.5	0.58	0.38
	Quadratic	Air	a = 0.000003 b = -0.000211 c = 0.003361	0.33 0.39 0.41	-165.9	0.42	0.44
Scaptotrigona luteipenis	Linear	Thorax	a = 0.000361 b = >-0.000001	<0.01* 0.02*	-514.7	0.42	0.17
	Quadratic	Thorax	a = >-0.000001 b = 0.000015 c = 0.000050	0.13 0.20 0.81	-515.3	0.58	0.24
	Linear	Air	a = 0.000356 b = -0.000002	<0.01* 0.05*	-513.2	0.24	0.13
	Quadratic	Air	a = -0.000001 b = 0.000031 c = -0.000186	0.05* 0.07 0.49	-515.5	0.76	0.25
Tetragonisca angustula	Linear	Thorax	a = 0.000479 b = -0.000007	<0.01 0.06	-458.1	0.33	0.12
	Quadratic	Thorax	a = 0.000001 b = -0.000088 c = 0.001814	0.08 0.06 0.02*	-459.5	0.67	0.21
	Linear	Air	a = 0.000464 b = -0.000007	<0.01* 0.08	-457.6	0.47	0.10
	Quadratic	Air	a = 0.000001 b = -0.000087	0.16 0.13	-457.8	0.53	0.17

			c = 0.001768	0.06			
Trigona fulviventris	Linear	Thorax	a = 0.000085 b = 0.000002	0.09 0.12	-512.9	0.08	0.09
	Quadratic	**Thorax**	**a = -0.000001 b = 0.000045 c = -0.000649**	**0.01* 0.01* 0.03***	**-517.9**	**0.92**	**0.28**
	Linear	Air	a = 0.000074 b = 0.000003	0.19 0.12	-512.9	0.08	0.08
	Quadratic	Air	a = -0.000001 b = 0.000057 c = -0.000810	0.01* 0.01* 0.02*	-517.7	0.92	0.27
Trigona muzoensis	Linear	Thorax	a = 0.000116 b = 0.000001	0.02* 0.58	-518.5	0.62	0.01
	Quadratic	Thorax	a = >-0.000001 b = 0.000021 c = -0.000213	0.34 0.33 0.54	-517.5	0.38	0.04
	Linear	Air	a = 0.000121 b = 0.000001	0.03* 0.67	-518.3	0.67	0.01
	Quadratic	Air	a = -0.000001 b = 0.000048 c = -0.000649	0.08 0.08 0.15	-519.7	0.33	0.11

B Species	Models	AIC	Akaike weights
Plebeia frontalis	Thorax-linear	-167.6	0.48
	Air-linear	-166.5	0.52
	Thorax-quadratic	-165.7	0.63
	Air-quadratic	-165.9	0.37
Scaptotrigona luteipenis	Thorax-linear	-514.7	0.68

	Air-linear	-513.2	0.32
	Thorax-quadratic	-515.3	0.47
	Air-quadratic	-515.5	0.53
Trigona fulviventris	Thorax-quadratic	-517.9	0.53
	Air-quadratic	-517.7	0.47

Table 3. Phylogenetic generalized least squares regression analysis statistics for the scaling of the difference between thorax and air temperature in the field and lab, Q_{10}, and the flight critical thermal maximum.

parameter	intercept	slope	std. err.	t	p	λ	p (for λ = 0)
thorax-air temp (field)	-0.767	0.759	0.177	4.289	0.008*	0.000	1.000
thorax-air temp (lab)	-1.093	2.015	0.235	8.583	0.001*	0.000	1.000
Q_{10}	1.077	0.017	0.069	0.243	0.820	0.000	1.000
CT_{max}	37.156	3.046	1.510	2.018	0.114	0.000	0.799

Figure 1. A) Log thorax- air temperature vs. log body mass of all individuals caught in the field using STRI's canopy crane in Fort San Lorenzo. Measurements were taken in sunny and cloudy conditions between 9am-3pm during the summer months. Different colors and shapes represent individuals of different species. B) Larger species had a greater elevation of thorax temperature above air temperature than smaller species (Table 3).

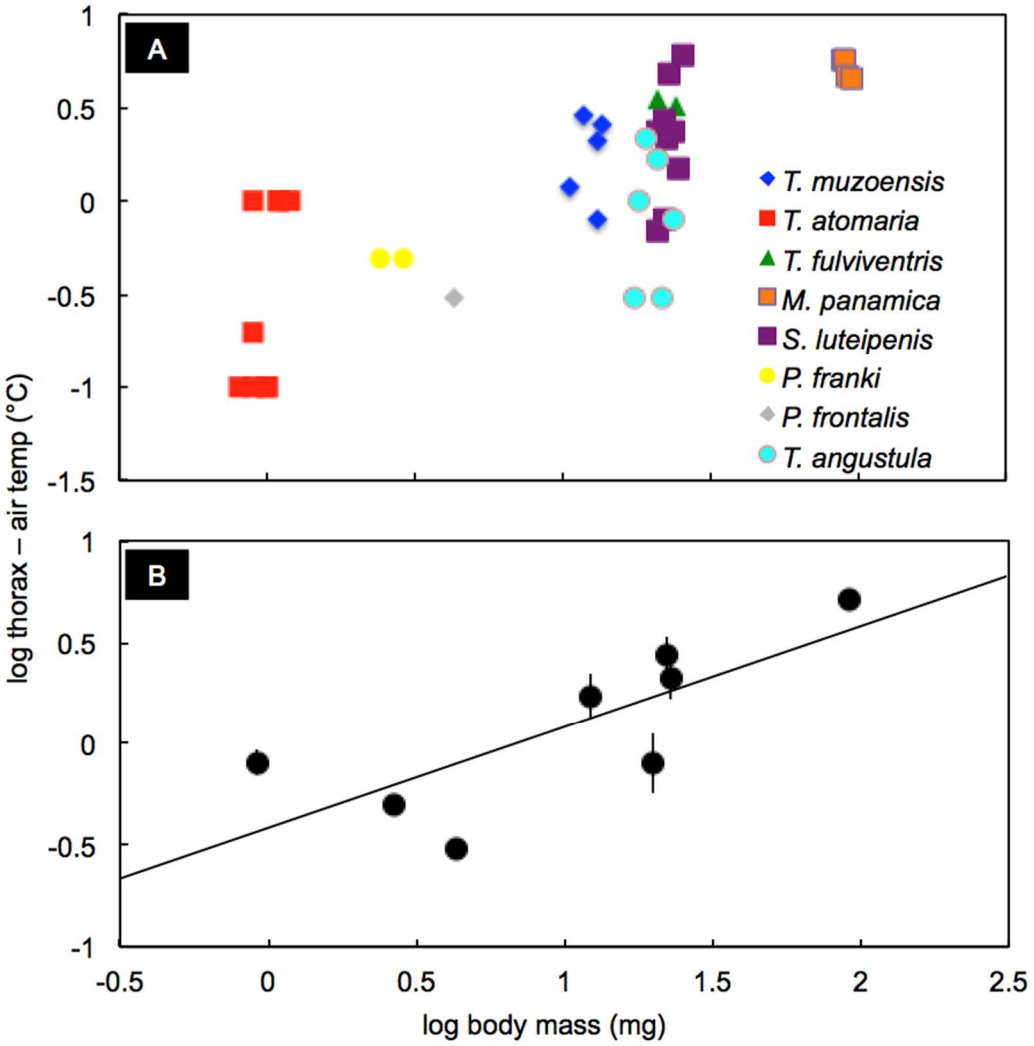


Figure 2. Average temperatures of leaves, flowers and air in the sun and shade. Leaves were always warmer than flowers and sunny locations were warmer than shady (two-factor ANOVA, Table 1).

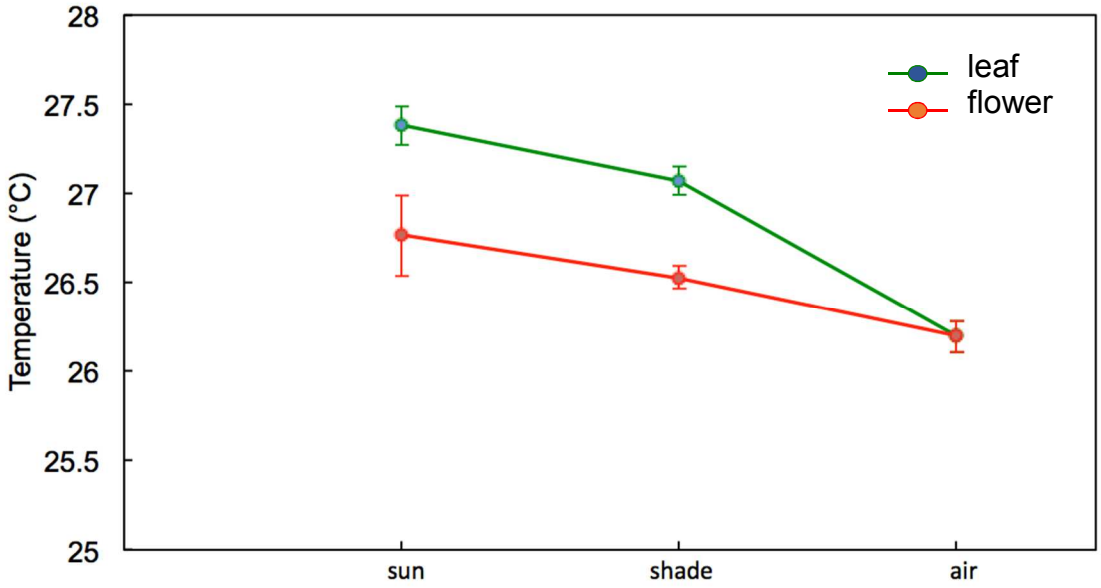


Figure 3. A-G) Thermal performance curves for stingless bee species in order of average body mass. For most species, there was no clear relationship between flight metabolic rate and air temperature. The metabolic rate of *P. frontalis* increases with increasing air temperature. The metabolic rate of *T. fulviventris* follows a quadratic function with peak flight metabolic rate at 39°C. The relationship between flight metabolic rate and air temperature was unclear in *S. luteipenis* with both linear and quadratic functions supported by AIC (linear and nonlinear model comparison in Table 2). F) The average difference between thorax and air temperature among species in flight metabolic rate measurements in the lab increased with body size (Table 3). I) Q_{10} did not vary with body size among stingless bee species between 25-35°C (Table 3).

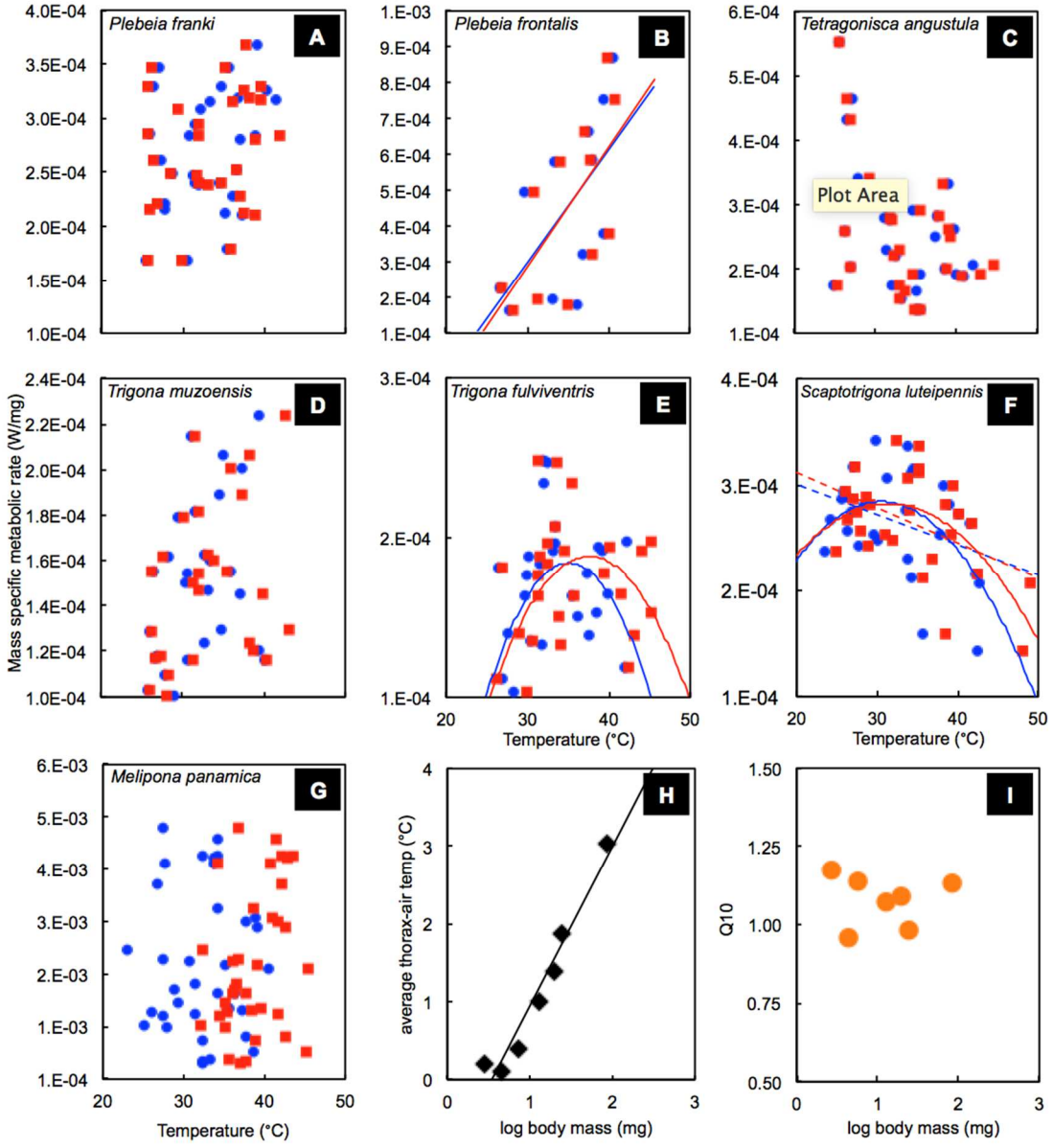


Figure 4. A comparison of observed thorax temperatures in the field (from June-August on average sunny and cloudy days) and the critical thermal maxima for flight found through respirometry in the lab indicates that bees are typically flying below their critical thermal maxima. This may be due to avoidance of flight during hot conditions.

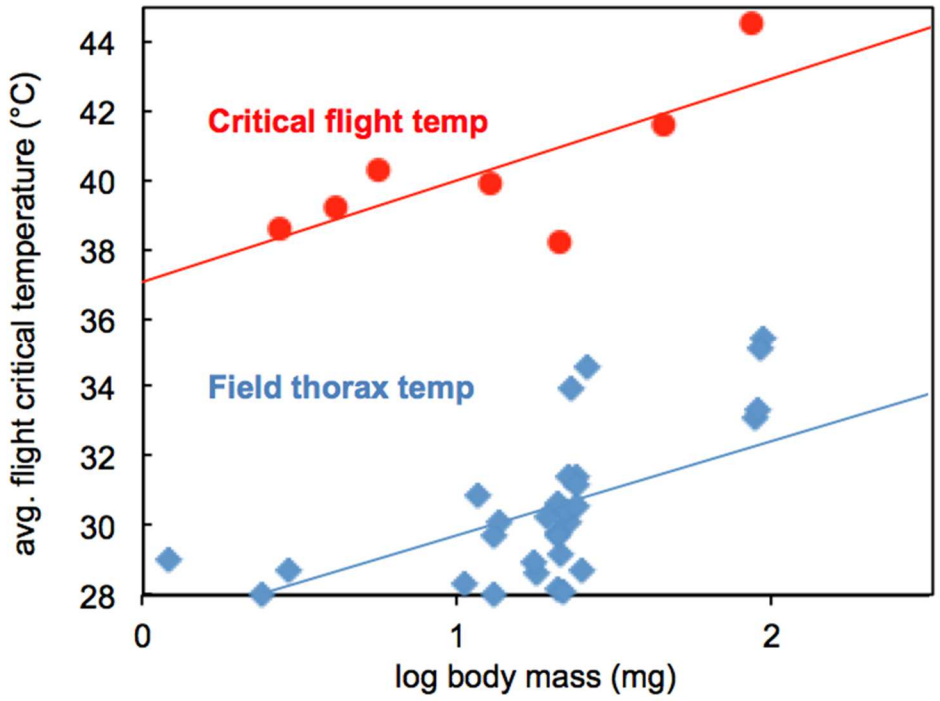
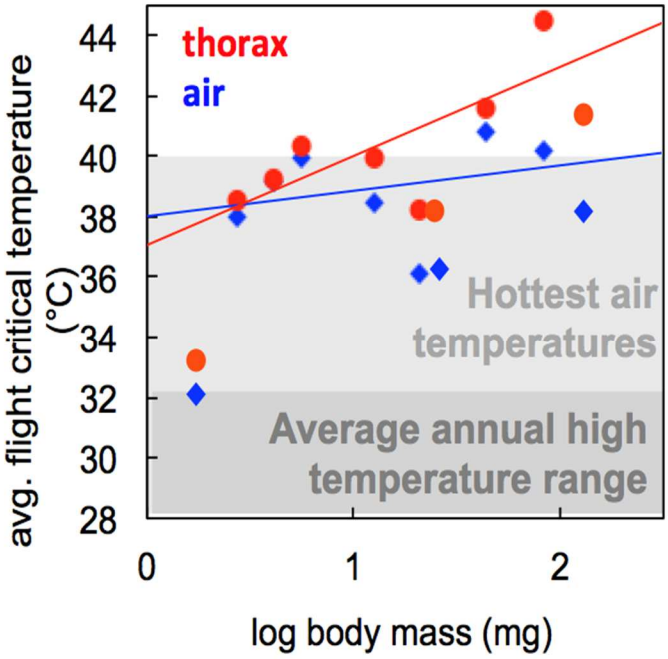


Figure 5. Flight critical thermal temperatures of stingless bees and corresponding air temperatures fall within the record and average annual high temperature range recorded for Panama. This suggests that current high temperatures causes thermal stress to some species in flight and that future climate warming will cause the number of species in thermal stress to increase.



REFERENCES

Angilletta, M.J. 2006. Estimating and comparing thermal performance curves *J. Thermal Biol.* 31 (7): 541-545.

Angilletta, M.J. 2009. *Thermal Adaptation:A theoretical and empirical synthesis.* (Oxford University Press Inc., New York, NY).

Bonner, J. 1979. *Why Size Matters.* (Princeton University Press, Princeton, NJ. (2006).

Bozinovic F, Bastias D.A., Boher F., Clavijo-Baquet S., Estay S.A., Angilletta M.J. 2011. The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiol. Biochem. Zool.* 84(6): 543-552.

Camargo, J.M.F. 2013. Historical biogeography of the Meliponini (Hymenoptera, Apidae, Apinae) of the Neotropical Region. *Pot Honey: a legacy of stingless bees.* 19-34 (P. Vit, S.R.M. Pedro, and D.W. Roubik. Springer Science + Business, New York).

Casey T.M. 1992. Biophysical ecology and heat exchange in insects. *Amer. Zool.* 32:225-237.

Chappell M.A. 1982. Temperature regulation of carpenter bees (*Xylocopa californica*) foraging in the Colorado desert of Southern California. *Physiol. Zool.* 55 (3): 267-280.

Chown S.L., Hoffmann A.A., Kristensen T.N., Angielletta M.J., Stenseth N.C., Pertoldi C. 2010. Adapting to climate change: a perspective form evolutionary physiology. *Clim. Res*. doi: 10.3354/cr00879

Chown S.L., Jumbam K.R., Sørensen J.G., Terblanche J.S. 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. *Func. Ecol.* 12:133-140.

Couvillon M.J, Fitzpatrick G., Dornhaus A. 2010. Ambient air temperature does not predict whether small of large workers forage in bumble bees (*Bombus impatiens*). *Psyche* 2010:536430.

Crozier L, Dwyer G. 2006. Combining population-dynamic and ecophysiological models to predict climate induced insect range shifts. *Am. Nat*. 167(6): 853-856.

Darveau, C.A., Suarez, R.K., Andrews, R.D., Hochachka, P.W. 2002. Allometric cascade as a unifying principle of body mass effects on metabolism. *Nature* 414, 166-170.

Dillon M.E., Frazier M.R. 2013. Thermodynamics constrains allometric scaling of

optimal development time in insects. *PLOS ONE.* 8(12): e84308.
Dillon M.E., Liu R., Wang G., Huey R.B. 2012. Disentagling thermal preference and the thermal dependence of movement in ectotherms. J. Thermal Biol. 37(8): 631-639.

Dillon M.E., Wang G., Huey R.B. 2010. Global metabolic impacts of recent climate warming. *Nature.* 467:704-707.

Dillon, M.E., Woods H.A., Wang G., Fey S.B., Vasseur D.A., Telemeco R.S., Marshall K., Pincebourde S. 2016. Life in the frequency domain: the biological impacts of changes in climate variability at multiple time scales. *Integrative and Comparative Biol.* 56,14-30. doi:10.1093/icb/icw024

Edeline E., Lacroix G., Delires C., Poulet N., Legeendre S. 2013. Ecological Emergence of thermal clines in body size. *Global Change Biol.* 19, 3062-3068.

Felsenstein, J. 1985. Phylogenies and the comparative method. *Am. Nat.* 125, 1–15.

Garamszegi, L.Z. 2014. *Modern Phylogenetic Comparative Methods and Their Application in Evolutionary Biology*. Springer-Verlag Berlin Heidelberg, Germany.

Gillooly, J., Brown, J., West, G.B., Savage, V.M., Charnov, E.L. 2001. Effects of size and temperature on metabolic rate. *Science.* 293: 2248-2251.

Harvey, P. H., Pagel, M.D. 1991. *The Comparative Method in Evolutionary Biology.* (Oxford University Press, New York, NY).

Harrison, J.F., Fewell, J.H., Roberts, S.P., Hall, H.G., 1996. Achievement of thermal stability by varying metabolic heat production in flying honeybees. Science 274, 88–90.

Harrison, J. F., Camazine, S., Marden, J. H., Kirkton, S. D., Rozo, A. and Yang, X. 2001. Mite not make it home: tracheal mites reduce the safety margin for oxygen delivery of flying honeybees. *J. Exp. Biol.*204,805 -814.

Heinrich B. 1972. Energetics of temperature regulation and foraging in a bumblebee *Bombus tericola kirby*. *J. Comp. Physiol.* 77(1): 49-64.

Heinrich B., 1980. Mechanisms of body temperature regulation in honeybees, *Apis mellifera* II. Regulation of thoracic temperature at high air temperatures. J. Exp. Biol. 85, 73–87

Huey R.B., Kearney M.R., Krockenberger A., Holtum J.A.M., Jess M., Williams S.E. 2012. Predicting organismal vulnerability to climate warming: roles, behavior, physiology and adaptation. *Phil. Trans. R. Soc. B*. doi:10.1098/rstb.2012.0005

Huey R.B. & Kingsolver J.G. 1989. Evolution of thermal sensitivity of ectotherm performance. *TREE* 4(5): 131-135.

Huey R.B., Kingsolver J.G. 2011. Variation in universal temperature dependence of biological rates. *Proc. Nat. Acad. Sci.* 108(26): 10377-10378.

Hulbert A.J. and Else P.L. 2000. Mechanisms underlying the costs of living animals. *Annu. Rev. Physiol.* 62: 207-235.

Jones J.C., Oldroyd B.P. 2007. Nest thermoregulation in social insects. *Adv. Insect Physiol.* DOI: 10.1016/S0065-2806(06)33003-2

Kingsolver, J. and Huey, R. 2008. Size, temperature, and fitness: three rules. *Evol. Ecol. Research* 10: 251-268.

Kingsolver, J.G, Woods H.A. 2016 Beyond thermal performance curves: Modeling time dependent effects of thermal stress on ectotherm growth rates. *Am. Nat.* 187, 283–294. DOI:10.1086/684786.

Klein A., Vaissiere B.E., Cane J.H., Steffan-Dewenter I., Cunningham S..A, Kremen C., Tscharntke, T. 2007. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B. DOI: 10.1098/rspb.2006.3721

Kremen, C., Williams, N.M., Bugg, R.L., Fay, J.P., Thorp, R.W. 2004. The area requirements of an ecosystem service: crop pollination by native bee communities in California. *Ecology Letters* 7: 1109-1119.

Lehmann, F. 1999. Ambient temperature affects free-flight performance in the fruit fly Drosophila melanogaster. *J. Comp. Physiol.* 169(3): 165-171

Lutterschmidt W.I., Hutchison V.H. 1997. The critical thermal maximum: history and critique. *Can. J. Zool.* 75(10): 1561-1574, https://doi.org/10.1139/z97-783

Mellanby K. 1932. The influence of atmospheric humidity on the thermal death point of a number of insects. *J. Exp. Biol.* 222-231

Menzel R., Greggers U. 1985. Natural phototaxis and its relationship to colour vision in honeybees. *J Comp. Physiol.* 157: 311 – 321

Michener, C.D. 2001. Comments on Minute Meliponini and the Male of the Genus Pariotrigona (Hymenoptera : Apidae) *J. Kansas Entomol. Soc.* 74, 231-236.

Norgate, M., Boyd-Gerny, S., Simonov, V. 2010 Ambient temperature influences Australian native stingless bee (*Trigona carbonaria*) preference for warm nectar. *PLoS ONE* 5(8): e1200.

Oyen K.J., Giri S., Dillon M.E. 2016. Altitudinal variation in bumble bee (*Bombus*) critical thermal limits. *J. Thermal Biol.* 59:52-57

Parmesan C., Ryrholm N., Stefanescu C., Hill J.K., Thomas C.D., Descimon H., Huntley B., Kaila L., Kullberg J., Tammaru T., Tennent W.J., Thomas J.A., Warren M. 1999. Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature* 399: 579-583.

Peters, R.H. 1983.*The Ecological Implications of Body Size*. (Cambridge University Press, New York).

Potter K.A., Woods H.A., Pincebourde S. 2013. Microclimatic challenges in global change biology. *Global Change Biol.*19, 2932–2939.

Reinhold, K. 1999. Energetically costly behaviour and the evolution of resting metabolic rate in insects. *Funct. Ecol.* 13: 217-224

Revell, L.J. 2010. Phylogenetic signal and linear regression on species data. Methods *Ecol. Evol*. 1: 319–329.

Roberts S.P. & Harrison J.F. 1998. Mechanisms of thermoregulation in flying bees. *Amer Zool*. 38:492-502.

Roberts S.P., Harrison J.F., Hadley N.F. 1998. Mechanisms of thermal balance in flying *Centris pallida* (Hymenoptera: Anthophoridae). *J Exp. Biol.* 202:2321-2331.

Roberts, S., Harrison, J. 1999. Mechanisms of thermal stability during flight in the honeybee *Apis mellifera. J. Exp. Biol.* 202: 1523-1533.

Roberts, S., Harrison, J., Hadley, N. 1998. Mechanisms of thermal balance in flying *Centris pallida (*Hymenoptera: Anthophoridae) *J. Exp. Biol.* 201: 2321-2331.

Roubik D.W. 1989. *Ecology and Natural History of Tropical Bees.* Cambridge University Press, Cambrdige, United Kingdom.

Roubik, D.W. 2000. Pollination system stability in Tropical America. *Conservation Biology.* 14 (5): 1235-1236.

Schleussner C., Lissner T.K., Fischer E.M., Wohland J., Perrette M., Golly A., Rogelj J., Childers K., Schewe J., Frieler K., Mengel M., Hare W., Schaeffer M. 2016. Differential climate impacts for policy-relevant limits to global warming: the case of 1.5°C and 2°C. *Earth Syst. Dynam.* 7, 327–351. www.earth-syst-dynam.net/7/327/2016/ doi:10.5194/esd-7-327-2016.

Schmaranzer, S. 2000. Thermoregulation of water collecting honey bees (*Apis mellifera*). *J. Insect Physiol.* 46(8): 1187-1194.

Stone G.N. 1994. Patterns of warm-up rates and body temperatures in flight in solitary bees of the genus *Anthophora*. *Funct. Ecol.* 8(3): 324-335.

Stone, B. and Willmer, P. 1989. Endothermy and temperature regulation in bees: a critique of 'grab and stab' measurement of body temperature. *J. Exp. Biol.* 143: 211-223.

Stone, G.N. 1994. Patterns of evolution of warm-up rates and body temperatures in flight in solitary bees of the genus *Anthophora*. *Functional Ecol.* 8(3): 324-335.

Terblanche J.S., Hoffmann A.A., Mitchell K.A., Rako L., le Roux P.C., Chown S.L. 2011. *J. Exp. Biol.* doi:10.1242/jeb.061283

Tsuji J.S., Kingsolver J.G., Watt W.B. 1986. Thermal physiological ecology of *Colias* butterflies in flight. *Oecologia.* 69(2): 161-170.

Vit, P., Pedro, S.R.M., and Roubik, D.W. 2013. *Pot Honey: a legacy of stingless bees.* Springer Publishers. New York, New York, United States.

Vollet-Neto A., Menezes C., Imperatriz-Fonseca V. 2015. Behavioural and developmental responses of a stingless bee (Scaptotrigona depilis) to nest overheating. *Apidologie* 46(4): 455-464.

Walczynska A., Kielbasa A., Sobczyk M. 2016. 'Optimal thermal range' in ectotherms defining criteria for tests of the temperature-size rule. *J. Thermal. Biol.* 60: 41-48.

West, G.B. 2002. Allometric scaling of metabolic rate from molecules and mitochondria to cells and mammals. *Proc. Natl. Acad Sci.* 99, 2473-2478.

Woods H.A., Dillon M.E., Pincebourde S. 2015. The roles of microclimatic diversity and of behavior in mediating the responses of ectotherms to climate change. *J. Thermal Biol.* 54:86-97.

Woods W.A., Heinrich B, Stevenson R.D. 2005. Honeybee flight metabolic rate: does it depend on air temperature? *J. Exp. Biol.* 1161-1173.

APPENDIX E

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