The physiological and life-history costs of parasitism: effect of the interaction between temperature and the ectoparasite *Varroa destructor* on *Apis mellifera*

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To my two beloved children Juan Andrés and Rafaela, my parents and family, friends, beekeepers and colleagues. All of them inspire me to continue and be better day by day even in the face of adversity.

Thanks for believing in me

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General Introduction

Anthropogenic activities have given rise to multiple challenges with potentially synergic detrimental effects for both wildlife and ecologically relevant species. Ongoing global change and a higher connectivity between geographic regions has exposed organisms not only to increasingly stressful temperatures, but also to invasive species and pathogens (Klein *et al*., 2017; Mandrioli, 2012). A case in point are honeybees. Indeed, the importance of *Apis mellifera* as a pollinator and the impact of this function on crop and seed production in agricultural ecosystems are crucial for human wellbeing (Klein *et al*., 2017). A decline has been noted in honeybee populations, especially of managed honeybees, since almost twenty years ago, as a result of different threats around the word (Kang *et al*., 2016; Le Conte *et al*., 2010; Nazzi and Le Conte, 2016; Neumann and Carreck, 2010; Ramsey *et al*., 2019; Ratnieks and Carreck, 2010; Requier *et al*., 2018; Staveley *et al*., 2014). Many putative causes have been proposed: pesticides, global change, new pathogens and pests, old pests made more virulent by a synergic effect with other pathogens, etc. (Cornelissen *et al*., 2019). It is known that the interaction between host and parasite implies energy costs for the hosts, reducing their survival rate and fecundity (Luong *et al*., 2017; Robar *et al*., 2011; Sadd and Schmid-Hempel, 2009).

The most widespread threat in beekeeping is the ectoparasitic mite *Varroa destructor* (Acari: Mesostigmata), responsible for the disease varroosis, which is present in almost every beekeeping country (Nazzi and Le Conte, 2016; OIE, 2019;

Ramsey *et al*., 2019). It causes more damage than all other known apicultural diseases and parasites (Emsen *et al*., 2015; Evans and Cook, 2018; Locke *et al*., 2014; Maggi *et al*., 2016). This ectoparasite affects individual bees and the whole colony because it feeds actively on the host, consuming the body fat (Ramsey *et al*., 2019) and the hemolymph of adult and immature bees (Ostermann *et al*., 2004; Richards *et al*., 2011). To date, however, the importance of this feeding activity on adult bees during their non-reproductive phase has not been determined (Nazzi and Le Conte, 2016). It also acts as a vector of pathogens like viruses, bacteria and fungi (Annoscia *et al*., 2012; Riveros *et al*., 2019).

A wide range of morphological and physiological changes have been reported in adult honeybees parasitized during their metamorphosis phase, such as a lower body weight, body and appendices deformities, decreased longevity, depression of the immune system, and reduction in hemolymphatic proteins (Annoscia, *et al*., 2012; Bowen-Walker and Gunn, 2001; Erban *et al*., 2019; Genersch *et al*., 2010; Gregory *et al*., 2005; Kralj and Fuchs, 2006; Lee *et al*., 2010; Schäfer *et al*., 2010; Yang *et al*., 2007). In the long term, this ectoparasite affects the Darwinian fitness of bees, causing important economic losses and degrading the functioning of the ecosystem (Boncristiani *et al*., 2012; Fries *et al*., 2006; Klein *et al*., 2017; Ramsey *et al*., 2019; Schmid-Hempel, 2008). Generally, when a host is parasitized it will allocate resources preferentially towards reproduction, even if this is at the expense of growth and survival (Agnew *et al*., 2000). Without treatment, most hives in

temperate climates die within 1 to 3 years (Ramsey and vanEngelsdorp, 2016; Rosenkranz *et al*., 2010).

Varroa reproduce inside the brood cells; when the mite feeds, it makes a wound in the brood bee's cuticle, used for feeding several times by both adult mites and offspring; mites feed on adult bees in the same way (Ramsey *et al*., 2018). To keep the wound open, the mite releases substances which inhibit the encapsulation process in the host (Kanbar and Engels, 2003).

Due to the success of *Varroa* in parasitizing honeybees, studies must focus on its physiological significance and its life history implications (Nazzi and Le Conte, 2016). Knowledge of changes in the host's energy allocation in response to parasites is crucial for understanding the impact of the parasite on both individuals and population levels (Garrido *et al*., 2016; Kutzer and Armitage, 2016; Schmid-Hempel, 2008; 2009). Studies of thermal biology in insect-parasite interactions have shown that resistance, host recovery, pathogen virulence and replication can be significantly altered by temperature (Schmid-Hempel, 2008; 2009), suggesting that the thermal environment could have profound implications for host/parasite dynamics and its co-evolution (Thomas and Blanford, 2003).

One way to measure integrative physiological variables is through the standard metabolic rate (SMR), which represents the energetic cost of living at a given temperature (Kovac *et al*., 2007; 2014). The metabolic rate in bees and other insects is affected by age (Kovac *et al*., 2007; Stabentheiner *et al*., 2003), race (Stabentheiner and Kovac, 2014), level of activity (Hartfelder *et al*., 2013), ambient

temperature (DeVries *et al*., 2016), body mass (DeVries *et al*., 2016; Stabentheiner and Kovac, 2014) and health status (Bordier *et al*., 2016; Kralj and Fuchs, 2010; Luong *et al*., 2017; Schmid-Hempel, 2008), among other factors. For instance, the energy expenditure of young bees at rest increases with ambient temperature several times below the values for highly active bees (Blatt and Roces, 2001; Kovac *et al*., 2007; Stabentheiner *et al*., 2003). According to Blatt and Roces (2001), a high level of activity could provoke a decrease in trehalose in the bees' hemolymph and an increase in glucose and fructose, reaching the maximal capacity of the fat body to synthesize trehalose.

Studies on energy have been conducted in healthy bees at different ages or activity levels and at different temperatures (Hartfelder *et al*., 2013) but without an acclimation process to warmer conditions. In addition, there is no report on the energetic cost of living in honeybees with any disease, except some inferences respecting bees infested with *Nosema ceranae* (Alaux *et al*., 2014; Bordier *et al*., 2016; Kralj and Fuchs, 2010; Naug, 2014).

Clearly, organisms living in large groups, as honeybees do, are particularly vulnerable to parasite transmission and disease (Klein *et al*., 2017; Kurze *et al*., 2016). Detrimental effects of parasitism on host fitness are usually attributed to parasite-associated disturbances to host energy budgets (Careau *et al*., 2010), sometimes provoking changes in the metabolic rate of the resting host (Robar *et al*., 2011). For instance, Luong *et al*., (2017) showed that when fruit flies (*Drosophila hydei*) were exposed directly to the ectoparasitic mite *Macrocheles*

muscaedomesticae, their energy expenditure increased by 35 % compared to flies with indirect contact, and to more than double the energy expenditure of uninfected flies. This is explained by the activation of the immune system, which might interfere with energy turnover or signaling mechanisms (Klein *et al*., 2017); the cost would result from maintaining defenses in a state of readiness (Bozinovic *et al*., 2013; Catalán *et al*., 2011; 2012a; 2012b; Moret and Schmidt-Hempfel, 2000; Schmid-Hempel, 2008), reducing another component of the host's fitness (Ardia *et al*., 2012; Otálora-Ardila *et al*., 2016). This trade-off between different fitness components leads to differences in how much defense is used (Frank and Schmid-Hempel, 2008). However, it is known that *Varroa destructor* activates the transforming growing factor beta or TGF-β-induced pathways in the bee to suppress wound healing and part of the immune response, and that the collective action of stressors intensifies these effects (Erban *et al*., 2019).

On the other hand, hemocytes are responsible for cell defense and for managing the nutritional elements extracted from the bees' diet and stored in the hemolymph (Szymaś and Jedruszuk, 2003). The total number of cells depends mainly on: the age of the bee (higher in younger individuals) (Schmid *et al*., 2008; Wilson-Rich *et al*., 2008); the quality of the diet (which affects the number and types of cells) (Alaux *et al*., 2010; Szymaś and Jedruszuk, 2003); and the bee's health status (Azzami *et al*., 2012; Belaid and Doumandji, 2010; Koleuglu *et al*., 2017; 2018; Marringa *et al*., 2014). As Hartfelder *et al*., (2013) mentioned, the protein content in hemolymph can provide valuable information on health status and correlated

processes (like disease or pest resistance), as well as information on the nutritional status of the bees (Cremonez *et al*., 1998).

But what happen if the host is constantly exposed to warmer temperatures? In this context, honeybee colonies around the globe are being exposed to increasingly higher temperatures due to global warming, which can have detrimental effects for multiple reasons. For instance, higher thermal averages and extremes may affect the honeybees' thermal performance, constrain their activity periods or increase water loss rates, all of which might affect survival and have a direct effect on colony stability (Annoscia *et al*., 2012; Klein *et al*., 2017; Mandrioli, 2012). In addition, energetic trade-offs associated with sublethal temperatures may have an impact on immune function and render these colonies more susceptible to infection (Schmid-Hempel, 2009) or, instead, increase the energy and water requirements of the hive, exacerbating the trade-offs between lifespan and immune function, leaving few resources available for disease resistance (Mandrioli, 2012). And finally, from a pathogen's or parasite's perspective, higher temperatures may have a positive effect on thermal performance and effectively promote or facilitate biological invasions by pests (Cornelissen *et al*., 2019). Needless to say, determining how changes in the thermal environment may affect the interaction between *Apis* and *Varroa*, as well as its impact on metabolic and thermal performance, is of paramount importance to determine how honeybee colonies might respond to different climate forecasts in the future (Kovac *et al*., 2007; Kovac *et al*., 2014).

Here, I hypothesized that bees maintained at higher acclimation temperatures would be more sensitive to *Varroa* infestation and exhibit more pronounced detrimental effects on their physiological variables caused by this ectoparasite than their counterparts maintained at less stressful temperatures. We also tested whether and by how much the total number of cells and the total protein amount in hemolymph as the survival rate in individual honeybees is affected by the number of mites and acclimation temperature. Thus, the aim of this research was to determine the impact and interaction between different parasitic loads of *Varroa* and acclimation temperature on some physiological functions on adult honeybee workers. I study adult bees because the evidence of detrimental effects of mites in this life stage remains limited and ambiguous (Nazzi and Le Conte, 2016), even though it has been speculated that the consumption of fat reserves by mites should significantly reduce energy storage and affect the immune response (Ramsey *et al*., 2019; Robar *et al*., 2011). The specifics aim were, to quantify the putative impact of thermal history in combination with a variable parasitic load on the energy expenditure, the individual survival rate, the amount of hemolymph cells and the total amount of lymphatic proteins in honeybees where everything else was maintaining equal.

Chapter 1

THE ENERGETIC AND SURVIVAL COSTS OF ECTOPARASITISM IN HONEYBEES

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Texto del capítulo 1

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SHORT TITLE

The cost of ectoparasitism by *Varroa destructor*

ABSTRACT

The ectoparasite *V. destructor* is the major threat to beekeeping (*A. mellifera*) worldwide, causing colony losses and reducing bees' productivity and pollinating capacity. Since parasitism produces high energy consumption in hosts, the aim of this study was to compare the effects of this ectoparasite on energy expenditure and survival rate in honeybees. Newborn bees were kept in chambers at 32 °C and 55 % humidity with food *ad libitum.* Individual bees were taken at random and grouped in three treatments: T_0 (no mites), T_1 (one mite) and T_2 (two mites). After the mites had fed on the bees, the metabolic rate (CO_2 production = VCO_2) was individually measured at 32 °C for three hours. We also measured survival rate, using the same groups for eight days. A significant effect of the number of mites on VCO_2 was found (T_0 = 3.14 ± 0.07 µLCO_2 min^{-1}, T_1 = 4.03 ± 0.03 µL CO_2 min^{-1} and T_2 = 6.44 ± 0.02 µL CO_2 min^{-1}, F= 25.81, p< 0.000001). In addition, the treatments affected significantly the bees' survival (F= 8.98, p= 0.002), with survival rates recorded of 57.5 % in T_0, 42.5 % in T_1 and 40.0 % in T_2. *V. destructor* clearly increases the energetic cost of living in bees and this effect may explain the reduction in survival rate.

Key words: Metabolic Rate. Survival. Parasitism. Energetic Costs.

1. INTRODUCTION

The importance of *Apis mellifera* as a pollinator and the impact of this function on crop and seed production in agricultural ecosystems are well known (Klein et al. 2007). A

decline has been noted in honeybee populations since more than twenty years ago, as a result of different threats around the word (Kang et al. 2016; Le Conte et al. 2010; Nazzi and Le Conte, 2016; Neumann and Carreck, 2010; Ramsey et al. 2019; Ratnieks and Carreck, 2010).

The most widespread threat in beekeeping is the ectoparasitic mite *Varroa destructor* (Acari: Mesostigmata), which is present in every beekeeping country except Mongolia, Lybia, Iraq, Uzbekistan, and some islands such as Trinidad and Tobago (OIE, 2019). It causes more damage than all other known apicultural diseases and parasites (Emsen et al. 2015; Evans and Cook, 2018; Locke et al. 2014; Maggi et al. 2016). This ectoparasite affects individual bees and the colony as a whole because it feeds actively on the host, consuming the body fat (Ramsey et al. 2019) and the hemolymph of adult and immature bees (Ostermann et al. 2004; Richards et al. 2011). It also acts as a vector of pathogens like viruses, bacteria and fungi (Annoscia et al. 2012). In the long term, this ectoparasite affects the Darwinian fitness of bees, causing important economic losses and degrading the functioning of the ecosystem (Boncristiani et al. 2012; Fries et al. 2006; Klein et al. 2017; Ramsey et al. 2019; Schmid-Hempel, 2008).

Generally, when a host is parasitized it will allocate resources preferentially towards reproduction, even if this is at the expense of growth and survival (Agnew et al. 2000). In the case of honeybees, a wide range of physical, physiological and behavioural changes has been described in adult bees parasitized during the pupal phase (Annoscia et al. 2012; Erban et al. 2019; Genersch et al. 2010; Kralj and Fuchs, 2006; Lee et al. 2010; Schäfer et

al. 2010; Yang et al. 2007). Mites feed on the brood during their reproductive phase (Nazzi and Le Conte, 2016) and during their phoretic phase (Ramsey et al. 2018; 2019); to date, however, the importance of this feeding activity on adult bees has not been determined (Nazzi and Le Conte, 2016). Without treatment, most hives in temperate climates die within 1-3 years (Ramsey and vanEngelsdorp, 2016; Rosenkranz et al. 2010).

Due to the success of *Varroa* in parasitizing honeybees, studies must focus on its physiological significance and its implications (Nazzi and Le Conte, 2016). Knowledge of changes in the host's energy allocation in response to parasites is crucial for understanding the impact of the parasite on both individuals and populations (Garrido et al. 2016; Kutzer and Armitage 2016; Schmid-Hempel, 2008; 2009). One way to measure integrative physiological variables is through the standard metabolic rate (SMR), which represents the energetic cost of living at a given temperature (Kovac et al. 2007; 2014). *Apis mellifera* is able to live under very different ecological conditions, keeping the internal temperature of the colony at 32-35°C (Kovac et al. 2007; 2014; Stabentheiner et al. 2010), or close to 27°C when the colony is broodless (Stabentheiner et al. 2010). The metabolic rate in bees and other insects is affected by age (Kovac et al. 2007; Stabentheiner et al. 2003), race (Stabentheiner and Kovac, 2014), level of activity (Hartfelder et al. 2013), ambient temperature (DeVries et al. 2016), body mass (DeVries et al. 2016; Stabentheiner and Kovac, 2014) and health status (Bordier et al. 2016; Kralj and Fuchs, 2010; Luong et al. 2017; Schmid-Hempel, 2008), among other factors. For instance, the energy expenditure of young bees at rest increases with the ambient temperature

from 0.212 µL O_2 min^{-1} at 10°C to 3.03 µL O_2 min^{-1} at 40°C; this is several times below the values for highly active bees (Blatt and Roces, 2001; Kovac et al. 2007; Stabentheiner et al. 2003). According to Blatt and Roces (2001), a high level of activity could provoke a decrease in trehalose in the bees' hemolymph and an increase in glucose and fructose, which its means that an upper limit in the capacity of the body fat to synthesize trehalose could be reached.

Studies on energy have been conducted in healthy bees at different ages or activity levels and at different temperatures (Hartfelder et al. 2013). However, there is no report on the energetic cost of living in honeybees with any disease, except some inferences respecting bees infested with *Nosema ceranae* (Alaux et al. 2014; Bordier et al. 2016; Kralj and Fuchs, 2010; Naug, 2014). Clearly, organisms living in large groups, such as honeybees inside hives, are particularly vulnerable to parasite transmission and disease (Klein et al. 2017; Kurze et al. 2016); the *Varroa* mite is the world's most frequent honeybee pest (Kang et al. 2016; Nazzi and Le Conte, 2016). Detrimental effects of parasitism on host fitness are usually attributed to parasite-associated disturbances to host energy budgets (Careau et al. 2010), sometimes provoking changes in the metabolic rate of the resting host (Robar et al. 2011). Luong et al. (2017) showed that when fruit flies (*Drosophila hydei*) were exposed directly to the ectoparasitic mite *Macrocheles muscaedomesticae*, their energy expenditure increased by 35% compared to flies with indirect contact, and to more than double the energy expenditure of uninfected flies. This is explained by the activation of the immune system, which might interfere with energy turnover or signalling

mechanisms (Klein et al. 2017); the cost would result from maintaining defences in a state of readiness (Bozinovic et al. 2013; Catalán et al. 2011; Catalán et al. 2012a; Catalán et al. 2012b; Moret and Schmidt-Hempfel, 2000; Schmid-Hempel, 2008), reducing another component of the host's fitness (Ardia et al. 2012; Otálora-Ardila et al. 2016). This trade-off between different fitness components leads to differences in how much defence is used (Frank and Schmid-Hempel, 2008). However, it is known that *Varroa destructor* activates the transforming growing factor beta or TGF-β-induced pathways in the bee to suppress wound healing and part of the immune response, and that the collective action of stressors intensifies these effects (Erban et al. 2019).

In this work we tested the effect of mites on the energy expenditure of individual honeybees under laboratory conditions. We also tested whether and by how much the survival rate in individual honeybees is affected by the number of mites.

2. MATERIALS AND METHODS

Honeybees (*Apis mellifera*) were kept in an apiary with six colonies located in Mediterranean agroecosystems of Central Chile (34°03'S; 70°41W); this apiary applies strict sanitary control for all diseases, especially against *Varroa destructor*, to ensure healthy bees with an infestation level of less than 2% of mites (two phoretic mites per one hundred worker bees). A second apiary with three colonies located at our laboratory (33°22'S; 70°36'W) is managed to have mites, so in this case, no treatment was applied,

and worker or drone brood production was stimulated to obtain a greater number of mites from each frame. During the late spring and summer of 2017-2018, we obtained worker and drone brood frames from the healthy colonies and moved them to a climate-controlled chamber at an ambient temperature (T_{Acclim}) of 32 ± 1.2°C, humidity 55 ± 5% and photoperiod L:D = 0:24 (Hartfelder et al. 2013). The worker brood remained in the chamber for the last 5 or 6 days as sealed brood. Emerged bees were kept in small, randomly grouped units of one hundred and fed with 50% sugar syrup solution and vitamins for 6-10 days before use in the study. We conducted *in vitro* assays, selecting 30 honeybees from different units and assigning them at random to each of three treatment groups: a) group T_0 (0 mites, control group), b) group T_1 (single honeybee treated with 1 mite), and c) group T_2 (single honeybee treated with 2 mites). At the same time, we preserved infested brood in a second chamber under the same conditions to rear mites according to Dietermann et al. (2013). Before starting each assay, enough mites were obtained to apply the treatments in each group of bees. The mites were kept in Petri dishes in a chamber for at least three hours to make them hungry (Dietermann et al. 2013).

2.1.Metabolic Rate

Honeybees were weighed in an analytical balance (±0.0001g; JK-180, Chyo, Kyoto) to the nearest mg and then one or two mites were placed directly on each bee with a brush. The bee was left alone and without movement in an Eppendorf tube in the chamber for

one hour to obtain direct, effective parasitization. In the case of the control group, the same procedure was followed but without mites. Ten resting bees per treatment were used, defining a resting bee as “no or only small visible signs of activity like small movements of antennae or single legs” (Kovac et al. 2007). After that, the rates of CO_2 production (VCO_2) were determined using an open-flow system consisting of a glass metabolic chamber as suggested by Lighton (2008) and Lighton and Halsey (2011). Each honeybee was placed in the metabolic chamber and this was placed in a temperature-controlled incubator at 32°C for 3 h. Air was drawn from the environment and CO_2 was scrubbed with a Drierite column; then VCO_2 was recorded continuously (Hartfelder et al. 2013; Lighton, 2008). The sample passed directly to the CO_2 analyzer (Sable system) with a flow of 150 ml/min. Data were transformed from percentage to volume per min and the total CO_2 production per individual was calculated with the EXPEDATA program (Sable Systems) (Chappell and Rogowitz, 2000; Hartfelder et al. 2013). With this information, a relationship was calculated for each treatment group between VCO_2 and number of mites (Kovac et al. 2007; Stabentheiner et al. 2012).

2.2.Survival.

To measure the survival probability, single bees were exposed to parasitization by 0, 1 or 2 mites in Petri dishes with supply of sugar syrup and water *ad libitum*. Each sample was kept in a chamber under the same conditions as before (T_{Acclim} = 32 ± 1.2°C, humidity = 55

± 5%, L:D = 0:24). The viability of the mite was recorded every day. Any mite that was in poor condition, or was not on the bee, was removed and replaced with a new one. The survival test was performed over a total of 8 days' observation with 10 bees per treatment and 4 repetitions each. A total of 40 bees were included in each treatment.

Statistical analyses were performed using the STATISTICA® (2001) version 6.0 statistical package for Windows®. Data were analyzed by one-way ANOVA and *a posteriori* Tukey test for multiple comparisons. Data fulfilled the assumptions of the tests. In the case of metabolic rate, the predictor variable was the number of mites and the dependent variable was the metabolic rate, VCO_2 ($\mu L min^{-1}$). Results are reported as mean ± 1 SD. In the case of survival data, a Kaplan and Meier test was performed first for each treatment to obtain the survival probability; a Log-Rank Test was applied subsequently to determine whether the differences between groups were significant.

3. RESULTS

3.1.Metabolic Rate.

The mean VCO_2 was 3.14 ± 0.07 $\mu LCO_2 min^{-1}$ in the control group, 4.03 ± 0.03 $\mu LCO_2 min^{-1}$ in the T_1 group and 6.44 ± 0.02 $\mu LCO_2 min^{-1}$ in the T_2 group. Thus the rate in bees infected with *Varroa* increased on average by 1.3 times in comparison to the control group. The metabolic rate in bees parasitized with two mites increased on average by 2.1 times (producing 3.30 $\mu LCO_2 min^{-1}$ more than the control group and 2.42 $\mu LCO_2 min^{-1}$ more

than the group treated with one mite) (see Table 1). The ANOVA test revealed that the number of mites significantly affected the VCO_2 in each bee ($F_{(1, 27)} = 25.81$, $p < 0.000001$) but the *a posteriori* Tukey's test revealed that the difference is significant only between T_0 and T_2 and between T_1 and T_2 (fig. 1).

3.2.Survival.

The results of survival over time and between groups are shown in Figure 2. After the Kaplan and Meier test, we observed that the number of *Varroa* mites reduced the survival probability of bees between groups. The survival probability at the end of the assay was 57.5% in the control group (T_0), 42.5% in T_1 and 40.0% in T_2 (fig. 3). The Log-Rank Test for the survival curves showed that there is no statistically significant difference ($p = 0.283$).

4. DISCUSSION

The effect of parasitism in its broadest sense involves metabolic changes in the host, reduction in the growth rate of juveniles and decreasing survival of hosts (Agnew et al. 2000; Careau et al. 2010). Our experiment illustrates at physiological and life-history levels how the parasite *Varroa destructor* plays a large, if not the largest, role in the high rate of colony losses registered around the world (Evans and Cook, 2018; Klein et al. 2017;

Ramsey and vanEngelsdorp, 2016; Ramsey et a. 2018; Requier et al. 2018). Our results show the effect, and its importance, of mites feeding on adult bees in the non-reproductive phase. Our main results can be summarized as follows: *Varroa* provokes an effect on the metabolism of resting bees (Frank and Schmid-Hempel, 2008; Luong et al. 2017; Sadd and Schmid-Hempel, 2009; Schmid-Hempel, 2008); as expected, the presence and number of mites significantly affects the energetic cost of living by increasing the metabolic rate in resting bees (Schmid-Hempel, 2008; Luong et al. 2017), as other bee parasites do (Alaux et al. 2014; Bordier et al. 2016; Kralj and Fuchs, 2010; Naug, 2014). These results contradict Robar et al. (2011), who did not detect an effect on the metabolic rate of hosts, although this does not reflect an absence of parasite-associated effects on the host's metabolic rate within systems. As mentioned above, the metabolic rate is affected by age, race (in some cases), activity level, temperature, body mass, immune system activation, and health (Bozinovic et al. 2013; Catalán et al. 2011; 2012a; 2012b; Hartfelder et al. 2013; Kovac et al. 2007; Stabentheiner et al. 2003; Stabentheiner and Kovac, 2014; Luong et al. 2017; Schmid-Hempel, 2008). The bees in this study were of the same age and race, kept at the same temperature, with no activity, similar body mass and no signs of disease at the beginning of the assays. Our results for the control group bees (T_0) were similar to those found in healthy, middle-aged bees (Blatt and Roces, 2001; Kovac et al. 2007; Kovac et al. 2014; Stabentheiner and Kovac, 2014); however, the energy cost increased in parasitized host bees as happens in chipmunks (~7.6% more for each parasite) or flies (~35% more) (Careau et al. 2010; Luong et al. 2017; Naug, 2014). No

reports were found for metabolic rates in bees infested by *V. destructor*, therefore no data were available for comparison; the metabolic rate in our bees was close to that of bees measured at 40°C by Stabentheiner et al. (2003). When the parasite affects a single bee or an entire colony, there is an energy cost which will vary according to infestation level, virus presence, nutrition, external stress factors, age, race, beekeeping management, immune system activation, etc. (Agnew et al. 2000; Careau et al. 2010; Emsen et al. 2015; Erban et al. 2019; Locke et al. 2014; Sadd and Schmid-Hempel, 2009; Rosenkranz et al. 2010). The final effect is a reduction in the fitness of the family, and the major colony losses explained by the presence of *V. destructor* (Kurze et al. 2016; Kang et al. 2016; Neumann and Carreck, 2010; Ramsey et al. 2019). Infestation induces high energy expenditure (increased energy turnover according to the number of parasites) and overuse of body fat (Blatt and Roces, 2001), generating an allocation of energy to activation of the immune system (Bozinovic et al. 2013; Catalán et al. 2011; Catalán et al. 2012a; Catalán et al. 2012b; Garrido et al. 2016; Luong et al. 2017; Moret and Schmid-Hempel, 2000; Schmid-Hempel, 2008); the quantities of some enzymes are decreased or increased in the wounding process and foraging compounds are injected during parasitism (Ardia et al. 2012; Koleoglu et al. 2017; Otálora-Ardila et al. 2016; Żółtowska et al. 2005). Additionally, parasitized bees weigh less, have a lower protein content and suffer a drastic reduction in longevity (Nazzi and Le Conte, 2016; Ramsey and vanEngelsdorp, 2016). When *Varroa* mites feed on bees, they extract lipids or fatty acids from the feeding wounds that they scrape into the host (Evans and Cook, 2018), affecting their reserves of

energy (Ramsey et al. 2018; Ramsey et al. 2019) or their energy-producing capacity (Blatt and Roces, 2001). As Figure 2 shows, the survival probability of honeybees is related with the number of mites, being lower when more parasites are present (Alaux et al. 2014; Bordier et al. 2016; Kralj and Fuchs, 2006; 2010; Naug, 2014). These results agree with previous reports on mortality rates in colonies parasitized by *Varroa destructor* (Lee et al. 2010; Schäfer et al. 2010; Rosenkranz et al. 2010).

Apart from our physiological data, no reports were found about the effect of *Varroa* on bees' individual survival. We adopted this approach because *V. destructor* is the only threat to honeybees that increases the risk of other pathogens, pesticides and poor resources in their environment (Evans and Cook, 2018). Overall, the number of mites increases the energetic cost of living in single honeybees, probably through activation of the immune system and direct damage to body fat, resulting in a survival cost. The metabolic cost of immune system activation is nearly 30 % in insects (Ardia et al. 2012) and could be higher in vertebrates (Careau et al. 2010; Otálora-Ardila et al. 2016). Thus, in our case, the immune response triggered by the *Varroa* mite entails a higher cost of maintenance and corresponding fitness (survival) costs.

It has previously been recommended in practical beekeeping to control *Varroa* mites due to the risk of virus transmission, keeping the number below a certain economic threshold. These findings confirm the importance of keeping *Varroa* numbers low because of the direct effects of parasitization.

5. CONCLUSIONS.

The energy cost for the bee is higher when it is parasitized, increasing by 1.28 and 2.05 times when the individual has one and two mites respectively. This means more energy expenditure related to the number of mites on each bee.

The survival probability compared with parasite-free bees is reduced by 15% and 17.5% when the bees are parasitized with one or two mites respectively.

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Authors' Contribution:

Both authors contributed to the writing, preparation and data analysis of this manuscript.

Conflicts of interest:

The authors declare no potential conflicts of interest in relation to the study in this manuscript.

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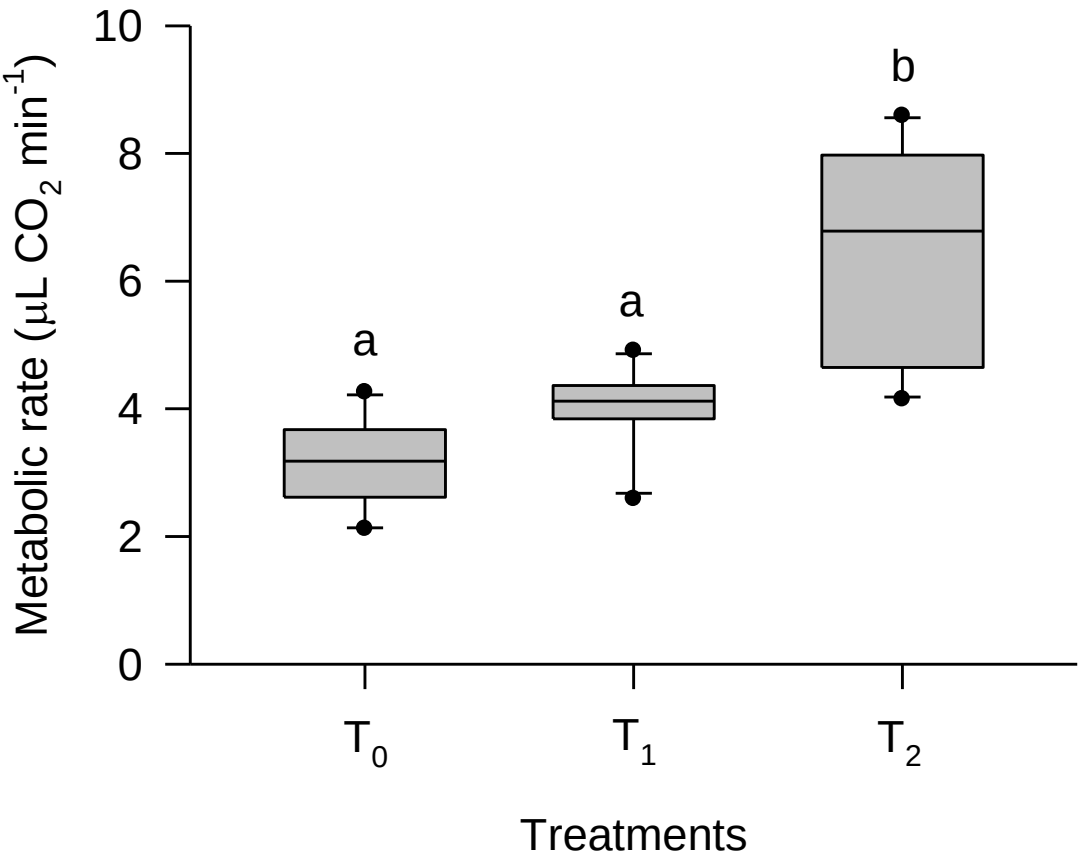

Figure 1. Effect of different numbers of the ectoparasite *Varroa destructor* on the metabolic rate of honeybees. Values are reported as mean ± 1 SD. Letters beside symbols refer to significant differences at $p < 0.05$ within each treatment group using a post hoc Tukey's test for multiple comparison. Groups were: T_0 = no mite, T_1 = one mite, T_2 = two mites.

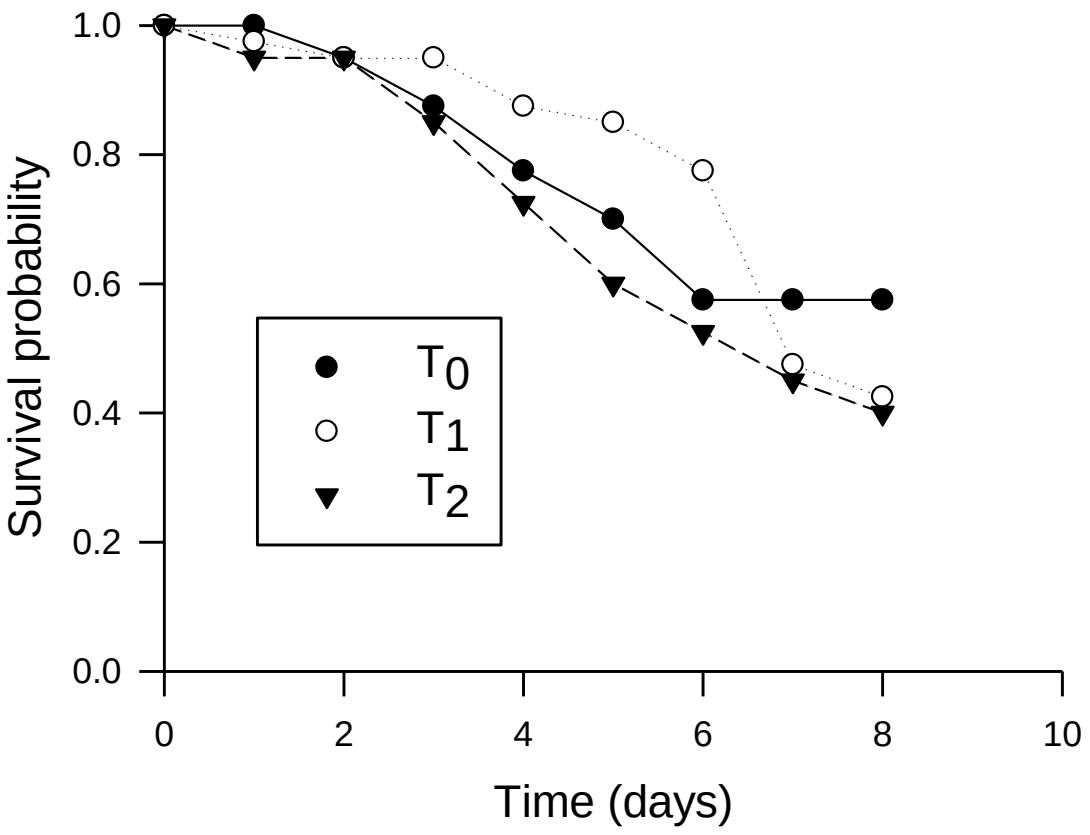

Figure 2. Survival rate between treatments using Kaplan- Meier Test with different load of *Varroa destructor* over time (days). Treatments were as follows: T_0 = no mite, T_1 = one mite, T_2 = two mites (see text).

Table 1. Metabolic rate in bees (VCO2 ($\mu L min^{-1}$)) when they are parasitized with different numbers of *Varroa* mites.

Treatments	N	Mean	SD	MAX	MIN
T0	10	3.14	0.07	4.26	2.12
T1	10	4.03	0.03	4.91	2.59
T2	10	6.44	0.02	8.59	4.15

T0: Control group; T1: Group with one mite; T2: Group with 2 mites; N: Number of bees tested; SD: Standard deviation; Max: Maximum value; Min: Minimum value.

Chapter 2

HEAT TOLERANCE, ENERGETICS AND THERMAL TREATMENTS OF HONEYBEES PARASITIZED WITH *VARROA*

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Texto del capítulo 2

Heat tolerance, energetics and thermal treatments of honeybees parasitized with *Varroa*

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Authors' contribution: All authors were involved in the design of the study. PA maintained the honeybee colonies, carried out the assays, analyzed the data and wrote the first draft of the manuscript; GR assisted with the heat tolerance assays and compilation of the data; ELR and FB assisted with analyses and contributed to the final draft.

Heat tolerance, energetics and thermal treatments of honeybees parasitized with *Varroa*

ABSTRACT

1. Ongoing global change affects both wildlife and economically relevant species, which are now subjected to combined challenges from climate change and higher exposure to pathogens. Honeybee colonies worldwide are under threat by higher temperatures and the ectoparasitic mite *Varroa destructor*, hence we studied the impact of these combined challenges in the thermal biology and energetics of *Apis mellifera*.
2. We estimated the heat tolerance and energy expenditure (CO_2 production, VCO_2) of honeybees acclimated to different temperatures (32 and 38 ºC) and subjected to different levels of parasitism (0, 1 and 2 mites). Heat tolerance was quantified employing thermal death time (TDT) curves describing how survival times vary as a function of temperature, which differed significantly between treatments.
3. Warm-acclimated uninfected bees exhibited a higher thermal tolerance than their cold-acclimated counterparts, but parasitism by *Varroa* resulted in a substantial drop in tolerance rendering TDT curves of parasitized bees virtually indistinguishable.
4. Accordingly, VCO_2 increased dramatically in parasitized bees (46.5% and 67.1% with 1 and 2 *Varroa*, respectively), suggesting that *Varroa* impinges on substantial costs on energy expenditure which, in combination with lower fat reserves due to parasitism, should have synergic effects on bees' survival and performance.
5. Result provide conclusive evidence of the detrimental impact of *Varroa* on heat tolerance that undermines potentially adaptive responses associated with thermal acclimation. Results also show that heat treatments are a realistic venue to control *Varroa*, and we discuss how TDT curves may be employed to optimize management strategies in this context.

Key words: *Apis mellifera*, Global warming, Metabolic rate, Survival, Thermal tolerance, *Varroa*, Varroosis.

INTRODUCTION

Anthropogenic activities have given rise to multiple challenges with potentially synergic detrimental effects for both wildlife and ecologically relevant species. Ongoing climate change and a higher connectivity between geographic regions has exposed organisms not only to increasingly stressful temperatures, but also to invasive species and pathogens (Klein Cabirol, Devaud, Barron, and Lihoreau, 2017; Mandrioli, 2012). Studies of thermal biology in insect-parasite interactions have shown that resistance, host recovery, pathogen virulence and replication can be significantly altered by temperature (Schmid-Hempel, 2008; 2009), suggesting that the thermal environment could have profound implications for host/parasite dynamics and co-evolution (see Thomas & Blanford, 2003). The reported population declines of honeybees (*Apis mellifera*) in different regions of the globe constitute a paramount example of this problem (Kang, Blanco, Davis, Wang, and DeGrandi-Hoffman, 2016; Maggi et al., 2016; Nazzi and Le Conte, 2016; Neumann and Carreck, 2010; Ramsey et al., 2019; Ratnieks and Carreck, 2010; Requier et al., 2018), with potentially important repercussions on crops and seeds productions in agricultural ecosystems (Mandrioli, 2012; Nazzi and Le Conte, 2016).

Several authors argue that these losses are caused by the ectoparasitic mite *Varroa destructor* (Acari: Mesostigmata), the most common pest in beekeeping that is responsible for the disease varroosis, present in virtually every country where the Western bee is found with few exceptions (Nazzi and Le Conte, 2016; Ramsey et al., 2019). This mite feeds directly from the host, consuming their fatty acids or lipids and the hemolymph from immature and adult bees (Richards, Jones, and Bowman, 2011; Ramsey et al., 2019), and also acts as a vector of viruses, bacteria and fungus (Annoscia, Del Piccolo, and Nazzi, 2012; Riveros et al., 2019). A wide range of morphological and

physiological changes have been reported in adult honeybees parasitized during their metamorphosis phase, such as a lower body weight, body and appendices deformities, decreased longevity, depression of the immune system, changes in the cuticular hydrocarbons profiles, and reduction in hemolymphatic proteins (Annoscia, Del Piccolo, and Nazzi, 2012; Erban, Sopko, Kadlikova, Talacko, and Haran, 2019; Lee, Moon, Burkness, Hutchison, and Spivak, 2010; Schäfer Ritter, Pettis, and Neumann, 2010). Without treatment, most of the colonies in temperate regions collapse in one to three years (Fries, Anton, and Rosenkranz, 2006; Ramsey and van Engelsdorp, 2016; Rosenkranz, Aumeier, and Ziegelmann, 2010). Overall, some estimations suggest that *Varroa* has provoked more damage to honeybee colonies than all other known honeybee diseases and parasites combined (Emsen, Hamiduzzaman, Goodwin, and Guzman-Novoa, 2015; Maggi et al., 2016; Evans and Cook, 2018).

On top of that, honeybee colonies around the globe are being exposed to increasingly higher temperatures due to global warming, which can have detrimental effects for multiple reasons. For instance, higher thermal averages and extremes may affect the honeybees' thermal performance, constrain their activity periods or increase water loss rates, all of which might affect survival and have a direct effect on colony stability (Annoscia, Del Piccolo, and Nazzi, 2012; Klein Cabirol, Devaud, Barron, and Lihoreau, 2017; Mandrioli, 2012). In addition, energetic trade-offs associated with sublethal temperatures may have an impact on immune function and render these colonies more susceptible to infection (Schmid-Hempel, 2009) or, instead, increase the energy and water requirements of the hive. And finally, from a pathogen's or parasite's perspective, higher temperatures may have a positive effect on thermal performance and effectively promote or facilitate biological invasions by pests (Cornelissen, Neumann, and Schweiger, 2019). Needless to say, determining how changes in the thermal environment may affect the interaction between *Apis* and *Varroa*, as well as its impact on metabolic and thermal performance, is of paramount importance to determine how honeybee colonies might respond to

different climate forecasts in the future (Kovac, Stabentheiner, Hetz, Petz, and Crailsheim, 2007; Kovac, Kâfer, Stabentheiner, and Acosta, 2014).

Here we address this issue, focusing on the impact of different parasitic loads of *Varroa* on the survival and energy expenditure of adult honeybee workers. We study adult bees because the evidence of detrimental effects of mites in this life stage remains limited and ambiguous (Nazzi and Le Conte, 2016), even though it has been speculated that the consumption of fat reserves by mites should significantly reduce energy storage and affect the immune response (Ramsey et al., 2019; Robar et al., 2011). Specifically, we quantified the putative impact of thermal history in combination with a variable parasitic load on heat tolerance and energy expenditure of honeybees everything else being equal. We hypothesized that bees maintained at higher acclimation temperatures would be more sensitive to *Varroa* infestation and exhibit more pronounced detrimental effects of this ectoparasite than their counterparts maintained at less stressful temperatures.

MATERIALS AND METHODS

Honeybees were kept in an apiary located in Mediterranean agroecosystems of Central Chile (34°03'S 70°41W) with six colonies. This apiary had a strict sanitary control for all diseases, especially against *Varroa*, to ensure that we have healthy bees with an infestation level under 2% of mites (two phoretic mites per one hundred of worker bees). A second apiary with three colonies, maintained in our laboratory (33°22'S 70°36'W), was employed as a source of *Varroa*. In this case no sanitary control was applied, and workers or drones brood production was stimulated to obtain a high number of mites from each frame. During the late Austral spring and summer of 2017 and 2018, we obtained worker and drone brood frames from the healthy colonies and moved them into two climatic chambers with ambient temperatures (T_a) of 32 or 38 ± 1.2 °C, humidity of 55 ± 5% and photoperiod of L:D = 0:24 (Hartfelder et al., 2013). The worker brood was maintained in the chambers as sealed brood for 5 or 6 days. Emerged bees were kept in small plastic

units grouped randomly (~100 individuals) and fed with 50% of sugar syrup solution and vitamins for 6 to 10 days before the experiments. In parallel, we maintained in a separate climatic chamber at 32 ± 1.2 °C the infested brood with *Varroa* (Dietermann et al., 2013).

To carry out the heat tolerance and metabolic assays with different loads of parasites (0, 1 or 2 *Varroa* per honeybee), we collected mites from infected brood and maintained them in Petri dishes for at least three hours at 32 ºC before transplanting them onto individualized healthy honeybees (below). This protocol ensured that mites would feed on their newly transferred host prior to measurements (Dietermann et al., 2013), resulting in an experimental design with control groups at each acclimation temperature (32 and 38 ºC) that were not infected, and experimental groups with contrasting parasitic loads.

Heat tolerance

We employed thermal death time (TDT) curves to estimate heat tolerance, as originally proposed by Rezende, Castañeda, and Santos (2014). This approach discriminates between the intensity and the duration of a thermal stress, which are confounded in assays with rising temperatures, indicating how organism might respond to an acute thermal challenge versus chronic exposition to less extreme temperatures (Rezende, Castañeda, and Santos, 2014). Succinctly, TDT curves can be described with the following relationship:

$$T = CT_{Max} - z \log_{10} t, \quad (1)$$

where T corresponds to the lethal temperature (ºC), CT_{Max} to the temperature resulting in death after a 1-min exposure (ºC), z to the temperature required to change the survival

time in one order of magnitude (ºC) and *t* the time to death (min). Note that CT_{Max} and *z* resemble the intercept and slope of a linear regression, and for sake of simplicity, a CT_{Max} = 40 ºC and *z* = 3 ºC would imply that an organism would tolerate 37 ºC for 10 min, 34 ºC for 100 min and so on (Rezende, Castañeda, and Santos, 2014).

We estimated TDT curves as implemented by Castañeda, Rezende, and Santos (2015), placing mites with the honeybees prior to assays. We collected adult bees younger than 9 days, weighed each individual in an analytical balance (± 0.1 mg; JK-180, Chyo, Kyoto), and placed one or two mites directly onto the bee with a brush and left them in an Eppendorf tube inside the climatic chamber for 1 h to ensure effective parasitism. For the control groups, we replicated the manipulation of the honeybees with the brush but did not place any mite onto them. Then, we measured heat tolerance for 60 individuals simultaneously in a water bath (46 × 35 × 35 cm^3) containing a rack with 4 rows × 15 columns of vials, with 15 individuals per treatment randomized within each bath. We used constant temperatures of 45, 47, 49 and 51 ºC, which resulted in assays lasting no more than 3 h, and two replicate baths per temperature. Water temperature was controlled by a programmable heating unit that also ensured proper water circulation (JULABO ED, JULABO Labortechnik, Seelbach, Germany). The behavior of each bee was recorded using a digital HD video camera (SONY HDRCX110E, Tokyo, Japan), and the time to death *t* was estimated as the period required for each individual to lose motor coordination or activity to cease. With this design, heat tolerance trials involved a total *n* = 720 honeybees (= 15 individuals × 4 measurement temperatures × 2 replicates × 2 acclimation temperatures × 3 parasitic loads) and the same number of *Varroa*.

Metabolic rate

Before metabolic trials, we weighed each individual and randomly assigned them to one of the three *Varroa* treatments as described above for the heat tolerance assays. Here, measurements involved ten bees per treatment, resulting in a total *n* = 60 (10 individuals

× 2 acclimations × 3 parasitic loads), and individuals were considered to be at rest only if "no or only small visible signs of activity like small movements of antennae or single legs" were observed (Kovac, Stabentheiner, Hetz, Petz, and Crailsheim, 2007). We measured rates of CO_2 production (VCO_2) in a glass metabolic chamber using an open-flow system (Sable Systems), following Lighton (2008), and Lighton and Halsey (2011). Each honeybee in the metabolic chamber was placed inside a temperature-controlled incubator, and measurements were performed for 3 h at the same temperature in which they were acclimated. Airflow was set to 150 ml/min, the CO_2 was scrubbed from the air with a Drierite column before entering the chamber and VCO_2 was continuously recorded (Lighton, 2008). Data were transformed from percentage to volume per min and total CO_2 production was calculated with EXPEDATA (Sable Systems).

Statistical analyses

Statistical analyses were performed using the STATISTICA® (2001) version 6.0 statistical package for Windows® operative system and R (https://cran.r-project.org). Analyses involved generalized linear models (GLM) to compare body size across acclimation temperatures, and then to compare heat tolerance and metabolic rates between treatments. Survival times at each temperature were compared employing a 2-factor ANOVA including acclimation and levels of parasitism as diagnostics for subsequent TDT analyses. Calculation of the TDT curve parameters was performed with separate linear regressions for each treatment, between measurement temperature T versus $\log_{10} t$ (Eq. 1), followed by the back-transformation CT_{Max} = – intercept/slope and z = 1/slope to ensure that analyses are performed with the appropriate minimum sums of squares (Rezende, Castañeda and Santos, 2014). Standard errors for CT_{Max} and z were estimated numerically from the error propagation of regression coefficient estimates, taking into consideration that slope and intercept are negatively correlated. We also quantified survival probability curves during heat tolerance challenges for all treatments as described

in Castañeda, Rezende, and Santos (2015). In these survival curves, the elapsed time *t* required for 50% mortality tends to exhibit the semi-logarithmic relationship with temperature *T* described by TDT curves (Eq. 1). Differences in heat tolerance across treatments were assessed with two complementary approaches. First, with a generalized linear model including $\log_{10} t$ as the dependent variable varying as a function of *T*, acclimation temperature, parasitic load (0, 1 and 2 *Varroa*) and $\log_{10}$ body mass:

$$\log_{10} t \sim T + T_{Acc} + Var + \log_{10} \text{mass} + (T \times T_{Acc}) + (T \times Var) \qquad (2)$$

Parasitic load *Var* was included as a factor (2 *df*) because preliminary analyses showed that the effects of the number of *Varroa* were non-additive. Differences in elevation between acclimation temperatures and levels of parasitism were estimated with main effects, whereas differences in slope were tested with the pairwise interaction between *T* and these terms (Eq. 2). Second, we compared the estimated 95% confidence intervals of parameters CT_{Max} and *z* and contrasted these results against the outcome of the GLM. For metabolic rates, we employed a similar GLM including only T_{Acc}, *Var* and $\log_{10}$ mass.

RESULTS

For both sets of honeybees employed for the heat tolerance and metabolic assays, acclimation temperature had a significant impact on body mass ($F_{1,718} = 617.7$, $P << 0.0001$) with a warm acclimation temperature resulting in smaller individuals (121.5 ± 14.7 mg at 32ºC and 93.5 ± 15.2 mg at 38 ºC for bees in the heat tolerance assays) (mean ± SD). Therefore, while analyses subsequent analyses are performed controlling for body mass, it is important to recall that the effects of acclimation temperatures in heat tolerance and metabolic rates can be partitioned into direct acclimation responses in heat tolerance and metabolic rates, and the indirect effects mediated by changes in body mass.

With regards to thermal tolerance, survival probability curves (Fig. 1) and comparisons between TDT curves (Fig. 2) show that heat tolerance is affected by both acclimation temperatures and levels of parasitism (Table 1). Interestingly, survival probability curves indicate that 1 or 2 *Varroa* have a conspicuous effects in mortality rates at less extreme temperatures, particularly at 45 and 47 ºC, whereas at higher temperatures the impact of heat stress prevail and survival curves obtained across honeybees subjected to different levels of parasitism become virtually indistinguishable (Fig. 1). These results are mirrored by the TDT curve analysis (Fig. 2), where we detected significant differences between elevation and slopes of curves as a function of acclimation temperature ($F_{1,711}$ = 8.67, P = 0.0033 and $F_{1,711}$ = 7.38, P = 0.0067 for the intercept and slope, respectively) and levels of parasitism ($F_{2,711}$ = 4.30, P = 0.014 and $F_{2,711}$ = 3.61, P = 0.028). Estimates of CT_{Max} indicate that TDT curves at 32 ºC are shifted downwards with respect to curves at 38ºC (Fig. 2), whereas estimates of z are generally lower in honeybees acclimated to 38 ºC and show that the increase in death times at less extreme temperatures is disproportionally higher in this group (Fig. 2). These results indicate that warm-acclimated honeybees exhibit a higher tolerance to nearly lethal and sublethal temperatures than their cold-acclimated counterparts. A closer inspection of this dataset indicates that *Varroa* parasitism reduces substantially survival times in bees acclimated at 38 but not at 32 ºC, and non-additive effects of multiple *Varroa* as suggested by preliminary analyses (Fig. 2). Interestingly, $\log_{10}$ body mass was highly significant in the GLM ($F_{1,711}$ = 34.3, $P = 7.31 \times 10^{-9}$), showing that larger individuals tended to collapse faster with a thermal challenge everything else being equal.

With regards to energy expenditure, VCO_2 was seemingly lower in the group acclimated to 38 ºC even after accounting for mass differences, suggesting that warm-acclimation results in a significant reduction in metabolism (Fig. 3). Accordingly, in the GLM allometric effects were weak albeit significant (scaling exponent b = 0.607 ± 0.332, $F_{1,55}$ = 3.35, 1-tailed P = 0.036) and thermal acclimation effects were negative ($F_{1,55}$ = 10.74, P = 0.0018). These results mirrored by analyses with the control group alone. While

the effects of parasitism in the full dataset were positive and significant ($F_{1,55}$ = 9.29, P = 0.0003), suggesting that parasitized honeybees exhibit a higher VCO_2 than control (Fig. 3), this effect pools the responses of the honeybees as well as the metabolic contribution of *Varroa*. Partitioning these effects is not entirely straightforward, particularly because differences in VCO_2 between treatments with 0, 1 and 2 *Varroa* show that effects are not additive: adjusted VCO_2 = 2.52 ± 0.23 µL CO_2/min, 3.69 ± 0.31 µL CO_2/min and 4.21 ± 0.35 µL CO_2/min, respectively (± SE). Assuming that the metabolism of *Varroa* is small to negligible given its small size when compared to adult bees, this would indicate that parasitism with 1 and 2 *Varroa* increases VCO_2 by, respectively, 46.5% and 67.1%.

DISCUSSION

Here we studied the effects of temperature acclimation and levels of parasitism in heat tolerance and energy expenditure of honeybees *A. mellifera*. Our results can be briefly summarized as follows. Bees acclimated to warmer temperatures exhibited a smaller size, higher thermal tolerance and decreased metabolic rates than their cold-acclimated counterparts. Contrasting responses between control and parasitized individuals suggest that warm-acclimated honeybees are more susceptible to the impact of *Varroa*, presumably due to their smaller size and more restricted energy reserves. The increase in energy expenditure detected in parasitized individuals was substantial and, in combination with the removal of fat deposits in parasitized individuals, is expected to have synergic detrimental effects. This might explain the high mortality rates observed during the beginning of the trials in parasitized honeybees, which are readily evident in the upper regions of the 45 ºC curves obtained following warm-acclimation (Fig. 1). Many bees were collapsing at the onset of the trials, most likely due to distress associated with parasitism rather than the heat shock per se. A poor physiological condition, combined with the rise in temperature and the metabolic challenge that this entails, likely explains this observation.

To our knowledge, this is the first estimation of TDT curves in healthy and parasitized honeybees, and results show that both acclimation history and *Varroa* have an impact on heat tolerance. Estimates of critical maximum temperatures obtained with ramping methods, where temperature increases at a constant rate, range between 44.6 to 51.8 ºC in different species of bees (Tan et al., 2005; Kovac, Kâfer, Stabentheiner, and Acosta, 2014; Hamblin, Youngsteadt, López-Uribe, and Frank, 2017), which fall within the range we estimated for an acute thermal stress. However, differences in TDT curves suggest that acclimation and *Varroa* effects are particularly relevant during chronic exposure at less extreme temperatures (Figs. 1 and 2). With regards to thermal acclimation, estimates of CT_{Max} and *z* for healthy individuals indicate that cold-acclimated bees can withstand 38 ºC for only 54 min (CT_{Max} = 53.2 ºC, *z* = 8.77 ºC) whereas their warm-acclimated counterparts can tolerate this temperature for 750 min (CT_{Max} = 53.7 ºC, *z* = 5.46 ºC) (calculations performed rearranging Eq. 1). While the later estimate is rather low considering that brood frames were maintained at 38 ºC, dehydration likely accounts for these lower survival times since bees had no access to food or water during TDT assays (Maynard-Smith, 1957; Rezende, Tejedo, and Santos, 2011). Consequently, this result combined with the smaller size of honeybees raised at 38 ºC suggests that this acclimation temperature already imposes some degree of sublethal stress, which might partly explain why TDT curves for warm-acclimated honeybees were more highly affected by *Varroa* (Fig. 2). For instance, while survival times estimated for a chronic exposure to 38 ºC is expected to decrease from 54 min to 36 min in cold-acclimated bees exposed to 1 *Varroa* (CT_{max} = 53.3 ºC, z = 11.3 ºC), in warm-acclimated bees estimates drop from 760 min to roughly 32 min (CT_{max} = 54.6 ºC, z = 11.0 ºC). We ignore why detrimental effects were apparently stronger in individuals with 1 instead of 2 *Varroa* (Fig. 2), but overall these results indicate that *Varroa* can have a disproportional effect on bees subjected to higher temperatures, hence parasitism and thermal stress may have synergic effects on survival and colony stability.

With regards to energy expenditure, resting VCO_2 in healthy individuals (Table 1) were similar to previous estimates of 2.14 µl/min at 32° C and 3.31 µl/min at 38° C (Kovac, Stabentheiner, Hetz, Petz, and Crailsheim, 2007; Kovac, Kâfer, Stabentheiner, and Acosta, 2014). As reported for some but not all host-parasite systems (see Robar et al., 2011), there was a marked increase in VCO_2 in the treatments with *Varroa* indicating that the acute response to parasitism in honeybees is energetically expensive. Visual inspection suggests that the non-additive effects of *Varroa* in VCO_2 were more pronounced in warm-acclimated bees, as observed for TDT curves, even though interactions between acclimation temperature and *Varroa* were not statistically significant possibly due to a restricted sample size in this experiment. Admittedly, the effects of body size, acclimation and measurement temperature are confounded, and it is considerably difficult to adequately tease them apart. Nonetheless, pairwise comparisons between treatments demonstrates that honeybees exhibit an enormous drop in metabolism in response to acclimation at 38 ºC, with VCO_2 decreasing even when thermal effects are not taken into account (Table 2). While mass-specific VCO_2 in non-parasite bees acclimated at 32 ºC and 38 ºC and measured at these temperatures represent a 26.7% decrease in energy expenditure (Table 2), this amounts is a 57.7 % drop after correcting for a Q_{10} = 2.5 and a 65.4% drop if differences in size are also considered. Despite the energy savings, it seems that this metabolic depression constitutes a stress response to elevated temperatures and, in the long run, would likely constraint activity and locomotor performance.

For logistic reasons, this experiment is constrained to acute responses to parasitism, acclimation to constant temperatures and we could not determine the outcome of the heat stress in mite's survival, which is crucial to assess the feasibility of heat treatment to control *Varroa* infestation. TDT curves have been employed to develop thermal treatments for pest control (Tang, Mitcham, Wang, and Lurie, 2007), and the available information in the literature suggests that this is a realistic possibility (Table 3). Heat treatments result in high *Varroa* mortality, hence TDT curves can be employed to find optimal combinations of temperature and exposure times to control *Varroa* and other

pathogens as long as the heat treatment does not negatively impact the honeybees (Fig. 4) (Bičík, Vagera, and Sádovská, 2016; Goras et al., 2015; Rosenkranz, 1987). Interestingly, despite the absence of standardized protocols across studies, multiple confounding factors and the uncertainty associated with many of the reported values (Table 3), published estimates of heat tolerance in *Varroa* vary in accordance with the framework employed here (Fig. 4) (Kablau, Berg, Härtel, and Scheiner, 2019; Komissar, 1985). A regression controlling for mortality as a factor (50% *versus* 80 – 100% mortality) results in a CT_{max} = 52.4 ºC and z = 4.19 ºC, which suggests that *Varroa* can tolerate heat stress for longer than the honeybees in our study (Fig. 2). This is not entirely surprising, however, because TDT curves reported here seem to underestimate heat tolerance of bees in the colony with access to water, food and shelter (see above), and most of the studies that we reviewed reported limited to negligible impact on brood and adult honeybees during thermal treatment (but see Harbo, 2000). Interestingly, higher honeybee mortality was generally observed during prolonged exposure to less extreme temperatures (48 to 76 h, see Harbo, 2000; Tabor and Ambrose, 2001), whereas treatments with an acute exposure to temperatures > 42ºC were generally less problematic for the bees. Consequently, our analyses strongly suggest that heat treatment may provide a viable solution to control *Varroa* and mitigate their impact on honeybees' populations and the ecosystem services that they provide. Thus, more detailed studies of thermal tolerance in both honeybees and *Varroa* within the hives are necessary for a characterization of TDT curves under more realistic conditions and to design effective management strategies to deal with parasite.

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FIGURES

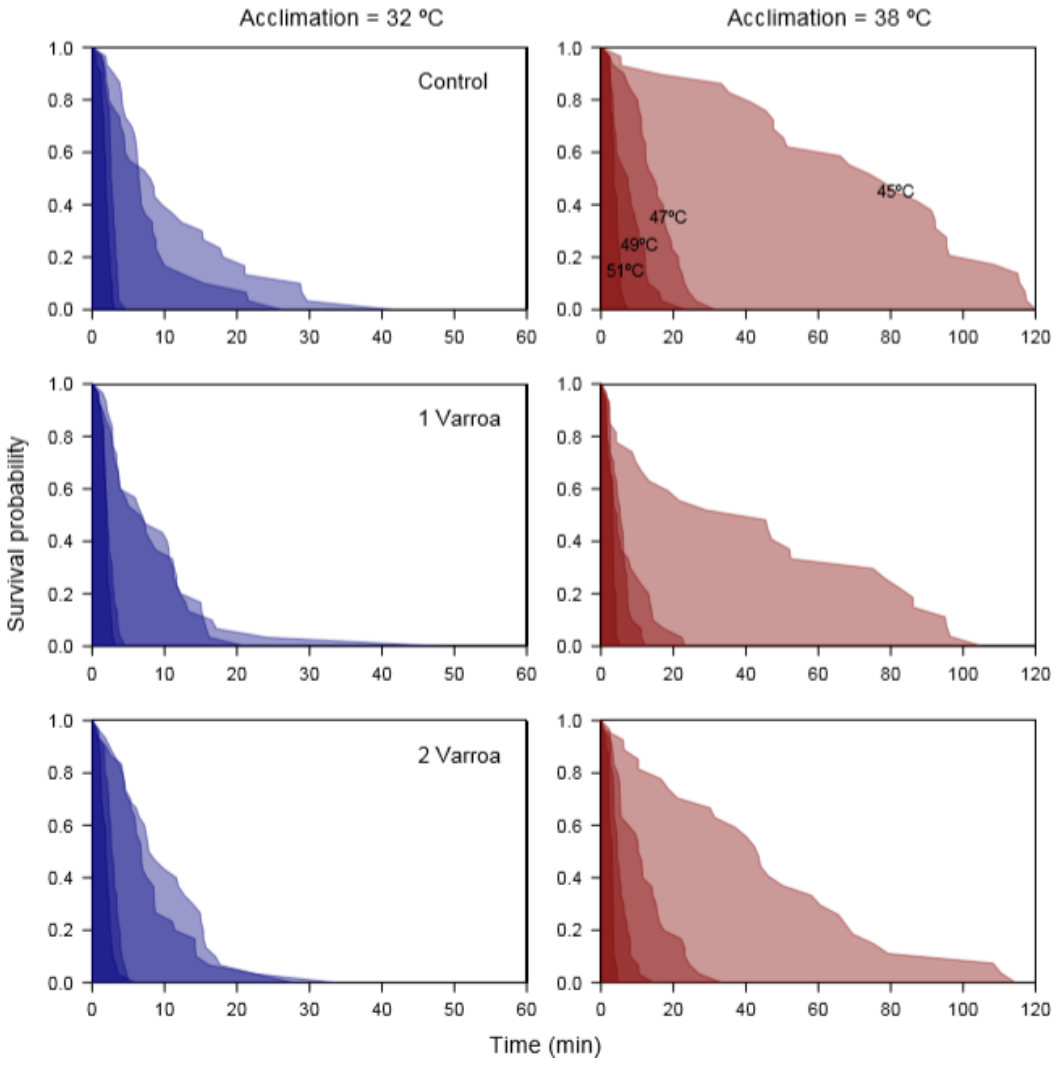

Figure 1. Survival curves obtained at different temperatures for honeybees acclimated to 32ºC and 38ºC and subjected to different levels of parasitism by Varroa (0, 1 and 2 mites). Measurements involved a total *n* = 720 individuals (*n* = 120 per panel).

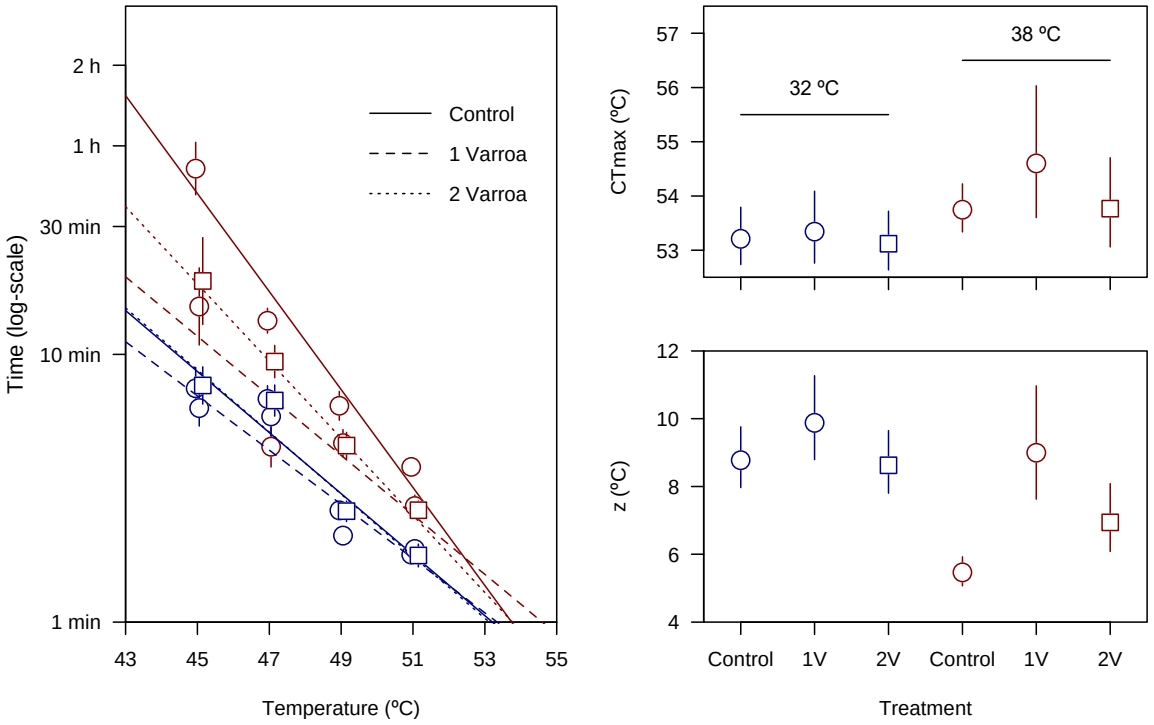

Figure 2. Heat tolerance in honeybees acclimated to 32ºC and 38ºC and exposed to different levels of parasitism by *Varroa*, expressed as thermal death time (TDT) curves. Parameters CT_{Max} and *z* represent, respectively, the thermal tolerance following an exposition of *t* = 1 min (i.e., the temperature that intercepts the abscissa) and the temperature difference required to increase *t* by one order of magnitude (see main text). Values are shown as mean ± SE.

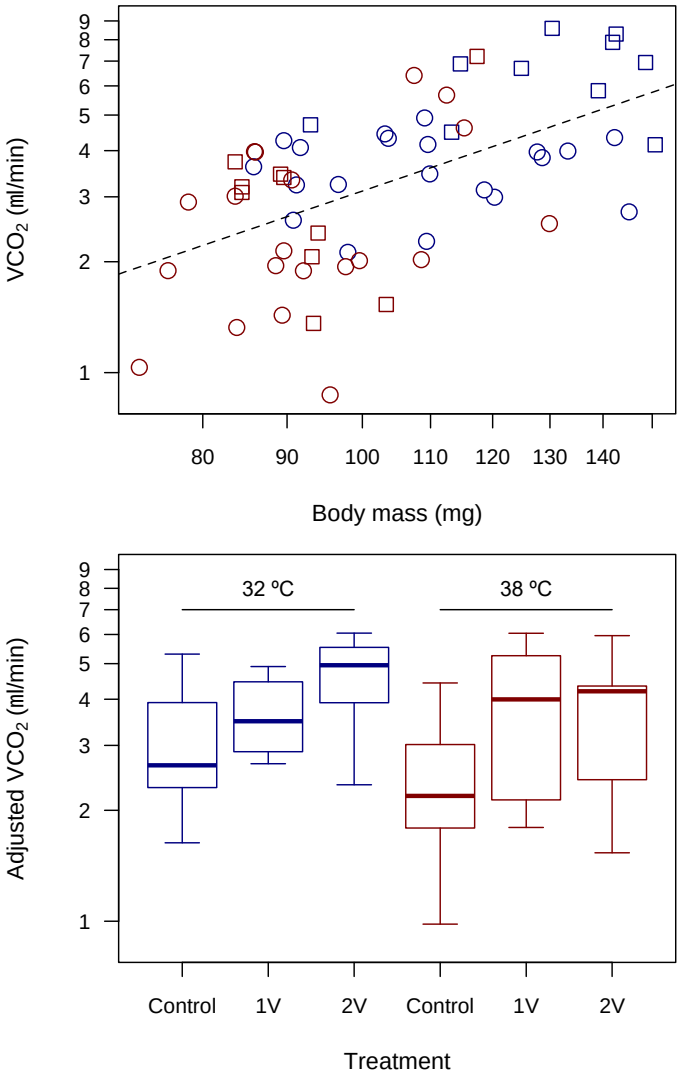

Figure 3. Metabolic rates in honeybees acclimated to 32ºC and 38ºC and exposed to different levels of parasitism by *Varroa*. For the boxplot adjusted estimates were calculated for a body mass of 103.5 mg. Symbols in the scatterplot as in Fig. 2.

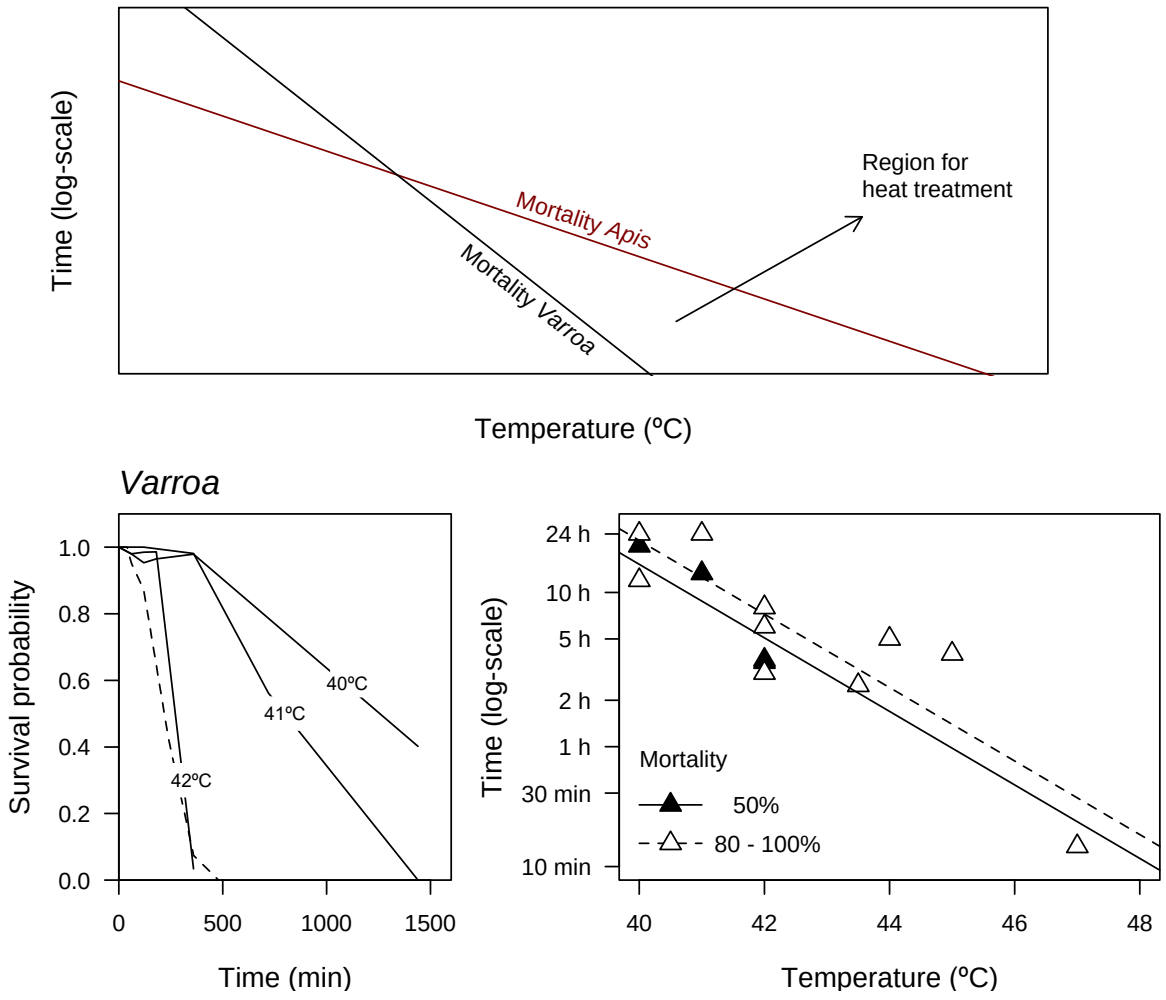

Figure 4. Heat treatment based on TDT curves of the honeybee and *Varroa* (based on Tang et al. 2007), applicable where the thermal tolerance of bees is higher than that of *Varroa.* Survival curves and mortality data from the literature suggest that thermal mortality in *Varroa* complies with expectations from TDT curves. Mortality of 50% was interpolated from survival curves, continuous and dotted lines represent different studies (see Table 3).

TABLES

Table 1. Survival times (min) employed to estimate the thermal death time (TDT) curves for different treatments (n = 180 for each temperature). Values are shown as mean ± SD and we report results from a 2-way ANOVA.

		Temperature			
Acclimation	***Varroa***	**45 ºC**	**47 ºC**	**49 ºC**	**51 ºC**
32ºC	0	11.4 ± 10.3	8.3 ± 5.8	3.1 ± 1.1	2.3 ± 1.0
	1	9.0 ± 9.2	8.1 ± 5.6	2.3 ± 1.3	2.4 ± 0.6
	2	10.2 ± 7.2	8.5 ± 6.4	3.2 ± 1.1	2.0 ± 1.0
38ºC	0	70.1 ± 36.3	15.2 ± 7.2	8.3 ± 5.2	4.4 ± 1.3
	1	39.1 ± 37.2	6.9 ± 6.4	5.5 ± 3.3	3.4 ± 1.1
	2	42.2 ± 34.1	12.1 ± 8.3	5.3 ± 3.3	3.2 ± 1.3
Acclimation	*d.f.* = 1,174	$F = 106.4$, $P < 0.0001$	$F = 10.24$, $P = 0.002$	$F = 77.49$, $P << 0.001$	$F = 83.94$, $P < 0.0001$
Varroa	*d.f.* = 2,174	$F = 7.11$, $P = 0.001$	$F = 6.48$, $P = 0.02$	$F = 4.81$, $P = 0.009$	$F = 5.55$, $P = 0.005$
Acclimation × Varroa	*d.f.* = 2,174	$F = 5.50$, $P = 0.005$	$F = 5.86$, $P = 0.003$	$F = 3.94$, $P = 0.02$	$F = 7.74$, $P < 0.001$

Table 2. Body mass and metabolic rate in honeybees from two thermal acclimation treatments subjected to different levels of parasitism (*n* = 10 for each group). Values are shown as mean ± SD.

Acclimation	***Varroa***	**Body mass (mg)**	**VCO_2 (μl/min)**	**Mass-specific VCO_2 (μl/min g)**
32ºC	0	108.3 ± 19.5	3.14 ± 0.66	0.030 ± 0.010
	1	112.1 ± 17.1	4.03 ± 0.63	0.036 ± 0.007
	2	129.9 ± 18.4	6.44 ± 1.60	0.050 ± 0.011
38ºC	0	88.3 ± 10.9	1.89 ± 0.69	0.022 ± 0.009
	1	100.1 ± 15.6	3.60 ± 1.64	0.036 ± 0.015
	2	93.3 ± 10.3	3.14 ± 1.65	0.033 ± 0.014

Table 3. Lethal temperatures and times reported for *Varroa* in the literature.

Temperature (ºC)	Time (min)	Mortality (%)	Reference
40	1205	50	Le Conte et al., (1990) ***
40	1440	97.4	Harbo (2000)
40	720	80 – 100	Rosenkranz (1987)
41	799	50	Le Conte et al., (1990) ***
41	1440	100	Le Conte et al., (1990)
42	219	50	Goras et al., (2016) ***
42	480	100	Goras et al., (2016)
42	180	95 – 100	Kablau et al., (2019)
42	212	50	Le Conte et al., (1990) ***
42	360	96.6	Le Conte et al., (1990)
40 – 47	150	100	Bičík et al., (2016)
44	300	80 – 100	Rosenkranz (1987)
45	240	80 – 100	Rosenkranz (1987)
47	12 – 15	95	Komissar (1985)

Only mortality rates comparable across studies were compiled (50% or ~ 90% mortality).

Reported ranges were averaged for analyses.

*** Interpolated from survival curves (see Fig. 4).

Chapter 3

THE INTERACTION BETWEEN AMBIENT TEMPERATURE AND *VARROA* PARASITISM DETERMINES CELL NUMBERS AND PROTEIN CONTENTS IN THE HEMOLYMPH OF WORKER HONEYBEES

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Texto del capítulo 3

The interaction between ambient temperature and *Varroa* parasitism determines cell numbers and protein contents in the hemolymph of worker honeybees

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Abstract

Varroa destructor is the major threat to European honeybees worldwide. To date no report has been found discussing the interaction between parasitism and ambient temperature; this is important in the new scenario of global warming, and to shed light on how global warming may or may not change the direct effects of parasites on host bees. The aim of this study was to determine the effect of the presence of Varroa mites on the number of cells and quantity of protein in hemolymph, and the individual survival rate at different acclimatization temperatures (T_{Acclim} = 32°C and 38°C). To do this, newborn bees were maintained in climate-controlled chambers at 32°C or 38°C and 55% humidity with food *ad*

libitum. Bees were grouped randomly in three treatments: T_0 (no mites), T_1 (one mite) and T_2 (two mites) at each acclimatization temperature. After the mites had fed on the bees, the number of cells and quantity of protein in hemolymph were determined and compared between treatments. The survival rates were estimated and compared using the same treatments and acclimatization temperatures for eight days. We observed a significant and detrimental effect of the load of mites on the number of cells, quantity of protein and survival both at hive temperature (32°C) and in warmer conditions (38°C); the higher temperature was more detrimental.

Key words: Proteins, hemolymph cells, mite, global warming, varroosis.

1. INTRODUCTION

The European honeybee is known worldwide as a major pollinator, improving crops, fruit and seed production (Kang et al. 2016; Nazzi and Le Conte, 2016). Global populations have been decreasing in recent decades, especially of managed honeybees (Ramsey et al. 2019; Requier et al. 2018; Staveley et al. 2014). Many putative causes have been proposed: pesticides, global change, new pathogens and pests, old pests made more virulent by a synergic effect with other pathogens, etc. (Cornelissen et al. 2019). It is known that the interaction between host and parasite implies costs for the hosts, reducing their survival rate and fitness due to the increase in their cost of living (Luong et al. 2017; Robar et al. 2011; Sadd and Schmid-Hempel, 2009). In this context, the ectoparasitic mite *Varroa destructor* is considered the principal enemy in beekeeping (Evans and Cook, 2018; Ramsey and vanEngelsdorp, 2016; Staveley et al. 2014) because of its general adverse effects on the host such as body mass reduction, shorter lifespan, immune incompetence, higher energy cost of both survival and thermal tolerance, etc. (Annoscia et al. 2012; Bowen-Walker and

Gunn, 2001; Erban et al. 2019; Gregory et al. 2005; Lee et al. 2010; Schäfer et al. 2010; Yang et al. 2007, Aldea et al. *in press*). Varroa reproduce inside the brood cells; when the mite feeds, it makes a wound in the brood bee's cuticle, used for feeding several times by both adult mites and offspring; mites feed on adult bees in the same way (Ramsey et al. 2018). To keep the wound open, the mite releases substances which inhibit the encapsulation process in the host (Kanbar and Engels, 2003).

Hemocytes are responsible for cell defense and for managing the nutritional elements extracted from the bees' diet and stored in the hemolymph (Szymaś and Jedruszuk, 2003). The total number of cells depends mainly on: the age of the bee (higher in younger individuals) (Schmid et al. 2008; Wilson-Rich et al. 2008); the quality of the diet (which affects the number and types of cells) (Alaux et al. 2010; Szymaś and Jedruszuk, 2003); and the bee's health status (Azzami et al. 2012; Belaid and Doumandji, 2010; Koleuglu et al. 2017; 2018; Marringa et al. 2014).

As Hartfelder et al. (2013) mention, the protein content in hemolymph can provide valuable information on health status and correlated processes (like disease or pest resistance), as well as information on the nutritional status of the bees (Cremonez et al. 1998).

In new climate change scenarios, increasing temperatures could exacerbate the trade-offs between lifespan and immune function, leaving few resources available for disease resistance (Mandrioli, 2012). Consequently, in this study we sought to estimate the effect of ambient temperature on the hemocyte cells and protein content of worker bees parasitized by *Varroa destructor,* and their survival rate, in laboratory conditions. The aim of the study was to determine the effect of the presence of Varroa mites on the number of cells and

quantity of protein in hemolymph, and the individual survival rate at different acclimatization temperatures (T_{Acclim} = 32°C and 38°C).

2. MATERIALS AND METHODS

2.1 Study area

Honeybees (*Apis mellifera*) were kept in an apiary with six colonies located in Mediterranean agroecosystems of Central Chile (34°03'S; 70°41'W); this apiary applies strict sanitary control for all diseases, especially against *Varroa destructor*, to ensure healthy bees with an infestation level of less than 1% of mites in adult bees. The sampling period was during the spring of 2019. A second apiary located in Universidad Mayor (33°22'S; 70°36'W), with three highly infested colonies (infestation level higher than 7%), was used as a source of mites for this research.

2.2 Experimental design

Between spring 2018 and spring 2019, we obtained worker and drone brood frames from the healthy colonies and moved them to two climate-controlled chambers at two different ambient temperatures (T_a): 32 ± 1.2°C and 38 ± 1.0°C; in both cases the humidity was 55 ± 5% and the photoperiod was L:D = 0:24 (Hartfelder et al. 2013). The worker brood stayed in the chamber as sealed brood for the last 3 or 5 days. Emerged bees were kept in random small groups of one hundred per unit and fed with 50% sugar syrup solution and vitamins for 1-10 days before use in the study, completing the acclimatization period. We then conducted *in vitro* assays, selecting 30 honeybees from different units, assigning them at

random to three treatment groups for each of the acclimation temperatures: a) group T_0 (0 mites, control group); b) group T_1 (each honeybee treated with 1 mite); c) group T_2 (each honeybee treated with 2 mites). At the same time, we preserved infested brood in a third chamber in the same acclimatization conditions to rear mites according to Dietermann et al. (2013). Before starting each assay, enough mites were obtained to apply the treatments in each group of bees. Mites were put in Petri dishes in a chamber for at least three hours to make them hungry (Dietermann et al. 2013).

2.3 Hemolymph samples

Hemolymph samples were obtained from the worker bees by severing the head and transferring the hemolymph into an Eppendorf tube with a micropipette for analysis (Gilliam and Shimanuki, 1971). The total number of hemolymph cells were counted according to Alaux et al. (2010). The number of hemocytes per microlitre (mm^3) of hemolymph was counted using a light contrast microscope (400x) with hemocytometer.

The total amount of protein was determined using the Bradford Test, by the standard method (Hartfelder et al. 2013). The total amount of protein in hemolymph was obtained in all groups (mg/mL).

2.4 Survival rates

To measure the survival probability, single bees were exposed to parasitization by 0, 1 or 2 mites in Petri dishes with a supply of sugar syrup and water *ad libitum*. Each sample was kept in a chamber under the same conditions as before (T_a = 32 ± 1.2°C; and T_a = 38 ±

1.0°C; humidity = 55 ± 5%, L:D = 0:24, in both cases). The viability of the mite was recorded every day. Any mite that was in poor condition, or was not on the bee, was removed and replaced with a new one. The survival test was performed over a total of 8 days' observation with 10 bees per treatment and 4 repetitions each. A total of 40 bees were included in each treatment.

2.5 Statistical analysis

Statistical analyses were performed using the STATISTICA® (2001) version 6.0 statistical package for Windows®. Data were analyzed by two-way ANOVA and *a posteriori* Tukey's test for multiple comparisons. Data fulfilled the assumptions of the tests. In the case of hemolymph samples, the predictor variables were the acclimatization temperature and the number of mites, and the dependent variables were the number of cells and the amount of protein. Results were reported as mean ± 1 SD. In the case of survival data, a Kaplan and Meier test was performed for each treatment to obtain the survival probability, and then a Log-Rank Test was applied by acclimatization temperature and group. Graphics were created with Sigma Plot version 11.0.

3. RESULTS

3.1. Hemolymph

Table 1 shows that the mean protein in hemolymph was higher in bees acclimatized at 32°C than in bees acclimatized at 38°C. Protein levels decreased when the mite was present; a clear effect was observed at 32°C, where the average protein level in T_1 was 47.38% of the

T_0 value, while in T_2 it was 27% of the T_0 value. At 38°C, the protein level was between 10.57 and 11.92 mg/mL in all three groups; the T_0 value (the lowest of the three) was 35.1% of the T_0 value at 32°C. The two-way ANOVA showed a significant effect of acclimatization temperature * number of mites on the total amount of protein in the hemolymph (see Fig. 1).

The same tendency was observed in the number of cells in hemolymph at 32°C (Table 1). The highest value was observed in the T_0 group at 32°C (1,210 cells/mm^3), and values decreased according to the number of mites. The number of cells in the T_0 group at 38°C was 16.7% of the T_0 value at 32°C. The results for cell numbers in the T_1 and T_2 groups at 38°C were not conclusive (see Fig. 2 and Discussion below).

3.2. Survival rate

The results of survival through time and between groups are shown in Figure 3. After the Kaplan and Meier test, we observed that the number of *Varroa* mites reduced the survival probability of bees between groups. The probability of survival at the end of the assay was 57.5% in the control group (T_0), 42.5% in T_1 and 40.0% in T_2 when the bees were acclimatized at 32°C; and 25% in T_0, 3.7% in T_1 and 15% in T_2, when T_{Acclim} = 38°C. The Log-Rank Test showed no significant effect between groups at the same acclimatization temperature, but a significant difference was found when the groups with the same numbers of mites were compared between the two acclimatization temperatures (Fig. 3; Table 2).

4. DISCUSSION

Our results show that the interaction between temperature and *Varroa destructor* has a high cost for the host, decreasing the number of cells and total protein in their hemolymph, and reducing the survival rate, see Figs. 1-3 (Sadd and Schmid-Hempel, 2009), reflecting a high energy cost of living (Luong et al. 2017; Schmid et al. 2008). These results are consistent with general information about the critical impact of this mite on honeybees (Evans and Cook, 2018; Ramsey and vanEngelsdorp, 2016; Ramsey et al. 2019).

We obtained an average of 1,210 cells/mm^3 in the control group at 32°C (normal hive temperature); this is one fifth of the number described by Alaux et al. (2010) and Szymaś and Jedruszuk (2003), but similar to that reported by Wilson-Rich et al. (2008). In the groups of bees treated at the same temperature (32°C), the number of cells decreased significantly in bees parasitized with one or two mites as compared to the control. These results agree with earlier information that the number of free cells in hemolymph is affected negatively when the mite is present because of the healing response (Belaid and Doumandji, 2010; Koleoglu et al. 2017; 2018; Wilson-Rich et al. 2008); however another possible reason is the direct immune suppression effect described in parasitized bees (Ardia, 2012; Gregory et al. 2005; Marringa et al. 2014; Yang and Cox-Foster, 2005; 2007). If the warmer *versus* colder acclimatization temperatures are compared, the number of cells in the T_0 group of bees at 38°C was 16.7% of the number at 32°C, and in the T_1 group it was 26.4% of the number in the colder group; however this tendency was lost when the host was parasitized by two mites, being 207 cells/mm^3 at 32°C *versus* 519 cells/mm^3 at 38°C. It is possible that when the bee is under a stressful temperature inside the hive, such as 38°C, it releases cells into the hemolymph only when needed (fewest cells

in T_0); such a need might be the presence of the Varroa mite. Alternatively, because the samples were taken just 60 min after parasitosis, the response is slower than under normal temperature conditions and is just beginning, while the aggregation process is not yet happening ($T_0 < T_1 < T_2$). The other possibility is that free cells were present in the hemolymph and the aggregation process was inhibited by a direct effect of the mite (Kanbar and Engels, 2003).

We found larger amounts of protein in bees than described by Wilson-Rich et al. (2008) in nurse bees (30.12 ± 8.04 mg/mL *versus* less than 10 mg/mL); the amount in our study was closer to the amount described by these authors for the pupae group. The amount of protein at 32°C decreased from the value with no mites in groups with one mite (47.12% of the T_0 value) and two mites (28.55% of the T_0 value), the value for T_2 being just over half that of T_1 (see Table 1 and Fig. 1). Although no other report has been found of decreasing protein in hemolymph, these results agree with previous reports that Varroa mites reduce the protein level in the tissues of the host (Bowen-Walker and Gunn, 2001). The Varroa mite is known to feed on body fat, and this tissue is the primary site of protein synthesis (Ramsey et al. 2019). In the case of the warmer acclimatization temperature, the amount of protein in hemolymph was similar between uninfested and infested bees, but in all cases was lower than the normal values obtained in the T_0 group at 32°C (Table 1, Fig. 1). No previous reports were found to compare between normal temperature and the effect on the total number of cells and protein in hemolymph when the ambient temperature is warmer, with or without mites on the bees.

The survival rate decreased significantly between the groups of bees at 32°C and at 38°C: with no mites (57.5% *vs.* 25%); with one mite (42.5% *vs.* 3.7%); with two mites (40.0% *vs.*

15%). Our results agree with Annoscia et al. (2012), who remarked on the detrimental effects on survival when abiotic conditions change the relationship between the host and the parasite (Sadd and Schmid-Hempel, 2008). We have no explanation as to why the survival rate is higher in the T_2 group than in T_1 at 38°C. As O'Connor and Bernhardt (2018) mentioned, changes in environmental factors such as temperature change the cost of parasitism in the host. We found that the survival rate is affected by temperature, but more strongly by the number of mites (see Fig. 3).

In conclusion, our results could explain the direct effect of warmer temperature on the number of cells, amount of protein and survival rate in bees with and without parasitization by Varroa. The interaction between temperature and number of mites is a trade-off between lifespan and immunity response (Mandrioli, 2012), but could also result, at least in part, from the changes in the metabolic rates of the host (Luong et al. 2017; O'Connor and Bernhardt, 2018; Sadd and Schmid-Hempel, 2009).

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Authors' Contribution:

Aldea prepared the draft of the manuscript, sampling and data analysis; Bozinovic and Sabat contributed to the writing, preparation and part of the data analysis of the final draft of the manuscript.

Disclosure statement

The authors declare no potential conflicts of interest in relation to the study in this manuscript.

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Table 1. Comparison between different numbers of *Varroa destructor* mites on the average amount of protein and number of cells in hemolymph of groups of bees according to acclimatization temperatures (T_{Acclim}).

Protein (mg/mL)				
Group	**T_{Acclim} 32°C**	**T_{Acclim} 38°C**	**ANOVA**	**p Value**
T_0	30.12 ± 8.04	10.57 ± 8.65	$F_{(2)} = 16.71$	< 0.000001
T_1	14.27 ± 6.7	11.91 ± 3.36	$F_{(1)} = 18.29$	< 0.000001
T_2	8.60 ± 1.98	10.82 ± 3.56	$F_{(2, 1)} = 18.63$	< 0.00007
N° of cells/mm^3				
Group	**T_{Acclim} 32°C**	**T_{Acclim} 38°C**	**ANOVA**	**p Value**
T_0	1,210 ± 255.67	202 ± 113.4	$F_{(2)} = 5.09$	0.0094
T_1	524 ± 284.1	386 ± 297.5	$F_{(1)} = 11.18$	0.0015
T_2	207 ± 102.2	519 ± 337.9	$F_{(2, 1)} = 26.14$	< 0.000001

Values are reported as mean ± 1 SD. Groups were as follows: T_0 = no mites, T_1 = one mite, T_2 = two mites.

Table 2. Comparison between different numbers of *Varroa destructor* mites on the survival rate of group of bees according with acclimatization temperatures (T_{Acclim}).

Comparison	**Survival Rate (%)**	**Log-Rank Test, p value**
T_{Acclim} 32°C	T_0 = 57.5 T_1 = 42.5 T_2 = 40.0	2.525, p= 0.283
T_{Acclim} 38°C	T_0 = 25.0 T_1 = 3.7 T_2 = 25.0	3.321, p= 0.190
T_0	32 *vs.* 38°C	9.694, p= 0.002
T_1	32 *vs.* 38°C	27.964, p< 0.001
T_2	32 *vs.* 38°C	6.335, p= 0.012

Groups were as follows: T_0 = no mites, T_1 = one mite, T_2 = two mites.

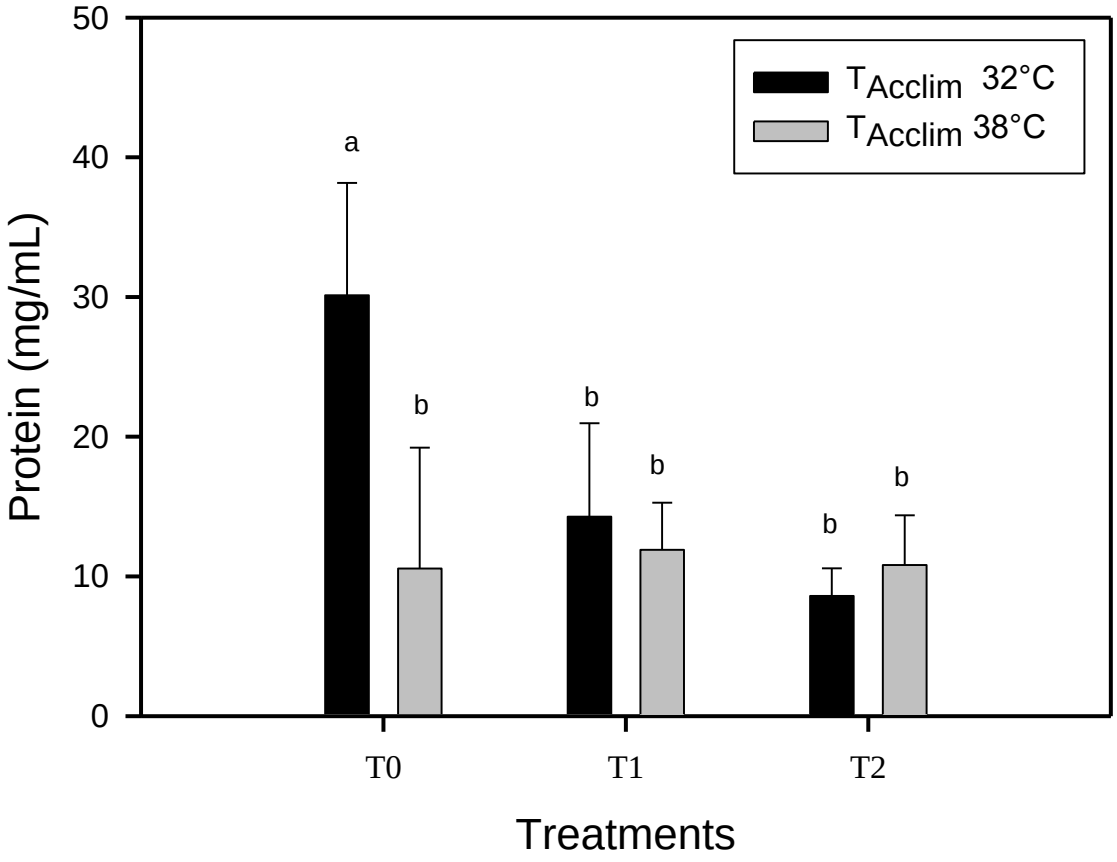

Figure 1. Effect of different numbers of the ectoparasite *Varroa destructor* on the average amount of protein in hemolymph between acclimatization temperatures. Values are reported as mean ± 1 SD. Letters beside symbols indicate significant differences $p < 0.05$ within each treatment group using a post hoc Tukey's comparison. Groups were as follows: T_0 = no mites, T_1 = one mite, T_2 = two mites.

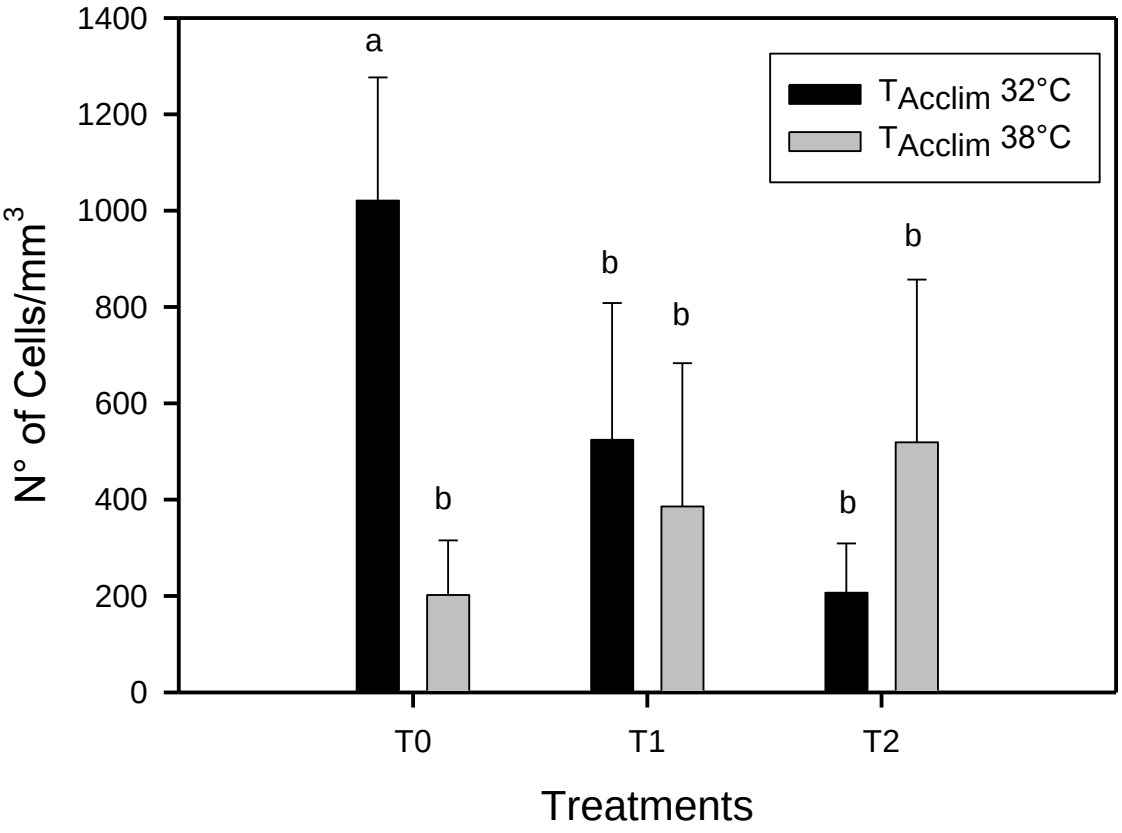

Figure 2. Effect of different numbers of the ectoparasite *Varroa destructor* on the average amount of hemocytes in hemolymph between acclimatization temperatures. Values are reported as mean ± 1 SD. Letters beside symbols refer to significant differences $p < 0.05$ within each treatment group using a post hoc Tukey's comparison. Groups were as follows: T_0 = no mites, T_1 = one mite, T_2 = two mites.

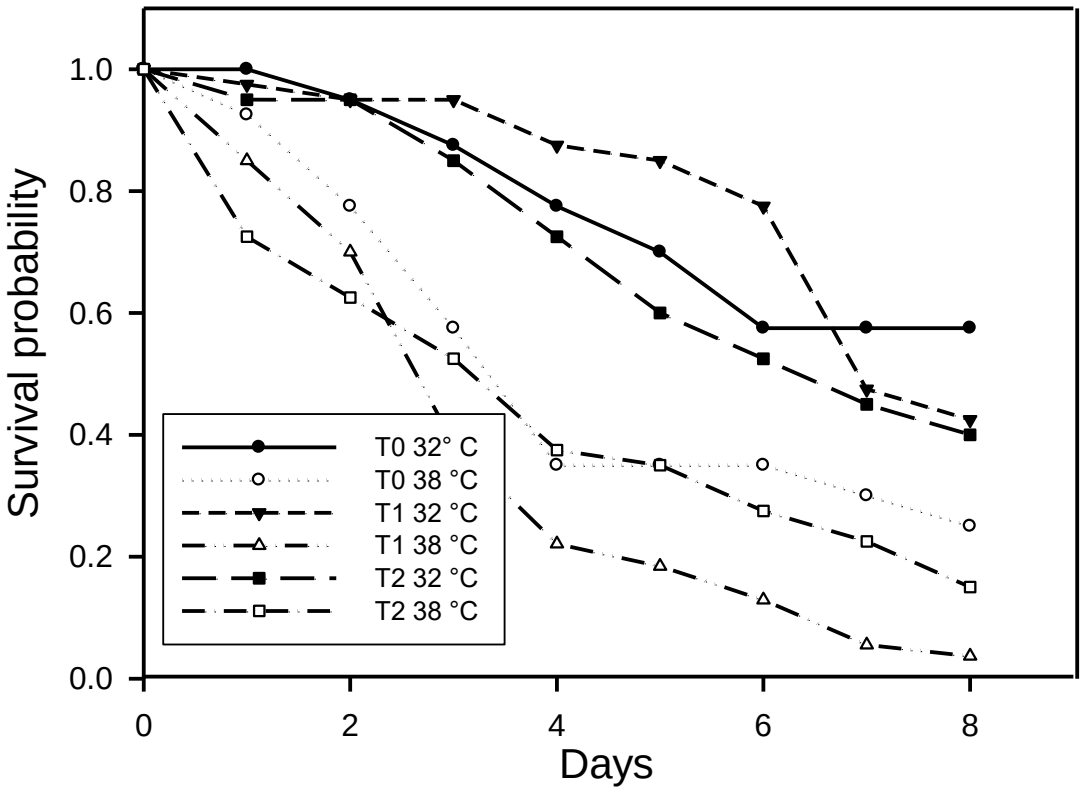

Figure 3. Survival rate between acclimatization temperatures and with different loads of *Varroa destructor* over time (days) using Kaplan-Meier Test. Groups were as follows: T_0 = no mites, T_1 = one mite, T_2 = two mites.

General Discussion

Here I tested the effects of temperature acclimation and levels of parasitism on energy expenditure, heat tolerance, individual survival capacity, total number of hemolymphatic cells and total amount of hemolymphatic proteins of workers of the honeybees *A. mellifera*. The effect of parasitism in its broadest sense involves metabolic and physiological changes in the host, reduction in the growth rate of juveniles and decreasing survival of hosts (Agnew *et al.,* 2000; Careau *et al.,* 2010). Our experiment illustrates at physiological and life-history levels how the parasite *Varroa destructor* plays a large, if not the largest, role in the high rate of colony losses registered around the world (Evans and Cook, 2018; Klein *et al.,* 2017; Ramsey and vanEngelsdorp, 2016; Ramsey *et al.,* 2018; Requier et al. 2018) and how is the direct effect of warmer temperatures in some physiological variables when the mite is present. Our results show the effect, and its importance, of mites feeding on adult bees in the non-reproductive phase. Our main results can be summarized as follows:

- *Varroa* provokes a direct effect on the metabolism of resting bees (Frank and Schmid-Hempel, 2008; Luong *et al.,* 2017; Sadd and Schmid-Hempel, 2009; Schmid-Hempel, 2008) when the acclimation was at cold (T_{Acclim}= 32 °C) and at warmer (T_{Acclim}= 38 °C) temperatures. As expected, in both acclimation temperatures, the presence and number of mites significantly affects the energetic cost of living by increasing the metabolic rate in resting bees (Schmid-Hempel, 2008; Luong *et al.,* 2017), as other parasites do (Alaux *et al.,* 2014; Bordier *et al.,* 2016; Kralj and Fuchs, 2010; Naug, 2014). These

results contradict Robar *et al.,* (2011), who did not detect an effect on the metabolic rate of hosts, although this does not reflect an absence of parasite-associated effects on the host's metabolic rate within systems. As was mentioned, the metabolic rate could be affected by age, activity level, temperature, body mass, immune system activation, and health status (Bozinovic *et al.,* 2013; Catalán *et al.,* 2011; 2012a; 2012b; Hartfelder *et al.,* 2013; Kovac *et al.,* 2007; Stabentheiner *et al.,* 2003; Stabentheiner and Kovac, 2014; Luong *et al.,* 2017; Schmid-Hempel, 2008). In this research all those factors were minimized to compare the effect of acclimation and number of parasites. Our results for the control group bees (T_0) in both temperatures were similar to those found in healthy, young and middle-aged bees (Blatt and Roces, 2001; Kovac *et al.,* 2007; Kovac *et al.,* 2014; Stabentheiner and Kovac, 2014); however, the energy cost increased in parasitized host bees as happens in chipmunks (~7.6 % more for each parasite) or flies (~35 % more) (Careau *et al.,* 2010; Luong *et al.,* 2017; Naug, 2014), indicating that the acute response to parasitism in honeybees is energetically expensive. With regards to energy expenditure, resting VCO_2 in healthy individuals were like previous estimates of 2.14 µl/min at 32 °C and 3.31 µl/min at 38 °C (Kovac *et al.,* 2007; 2014). Nonetheless, pairwise comparisons between treatments demonstrates that honeybees exhibit an enormous drop in metabolism in response to acclimation at 38 ºC, with VCO_2 decreasing. While mass-specific VCO_2 in non-parasite bees acclimated at 32 ºC and 38 ºC and measured at these temperatures represent a 26.7 %

decrease in energy expenditure, this amounts is a 57.7 % drop after correcting for a Q_{10} = 2.5 and a 65.4% drop if differences in size are also considered. No reports were found for metabolic rates in bees infested by *V. destructor*, therefore no data were available for comparison. When the parasite affects a single bee or an entire colony, there is an energy cost which will vary according to infestation level, virus presence, nutrition, external stress factors, age, race, beekeeping management, immune system activation, etc. (Agnew *et al.,* 2000; Careau *et al.,* 2010; Emsen *et al.,* 2015; Erban *et al.,* 2019; Locke *et al.,* 2014; Sadd and Schmid-Hempel, 2009; Rosenkranz *et al.,* 2010).

- Contrasting responses in heat tolerance between control and parasitized individuals suggest that warm-acclimated honeybees are more susceptible to the impact of *Varroa*, presumably due to their smaller size and more restricted energy reserves. The increase in energy expenditure detected in parasitized individuals was substantial and, in combination with the removal of fat deposits in parasitized individuals, is expected to have synergic detrimental effects. This might explain the high mortality rates observed during the beginning of the trials in parasitized honeybees, which are readily evident in the upper regions of the 45 ºC curves obtained following warm-acclimation. Many bees were collapsing at the onset of the trials, most likely due to distress associated with parasitism rather than the heat shock per se. A poor physiological condition, combined with the rise in temperature and the metabolic challenge that this entails, likely explains this observation. To our

knowledge, this is the first estimation of TDT curves in healthy and parasitized honeybees, and results show that both acclimation history and *Varroa* have an impact on heat tolerance. Estimates of critical maximum temperatures obtained with ramping methods, where temperature increases at a constant rate, range between 44.6 to 51.8 ºC in different species of bees (Tan *et al.,* 2005; Kovac, *et al.,* 2014; Hamblin *et al.,* 2017), which fall within the range I estimated for an acute thermal stress. However, differences in TDT curves suggest that acclimation and *Varroa* effects are particularly relevant during chronic exposure at less extreme temperatures. With regards to thermal acclimation, estimates of CT_{Max} and *z* for healthy individuals indicate that cold-acclimated bees can withstand 38 ºC for only 54 min (CT_{Max} = 53.2 ºC, *z* = 8.77 ºC) whereas their warm-acclimated counterparts can tolerate this temperature for 750 min (CT_{Max} = 53.7 ºC, *z* = 5.46 ºC). Consequently, this result combined with the smaller size of honeybees raised at 38 ºC suggests that this acclimation temperature already imposes some degree of sublethal stress, which might partly explain why TDT curves for warm-acclimated honeybees were more highly affected by *Varroa*. I ignore why detrimental effects were apparently stronger in individuals with one instead of two *Varroa*, but overall these results indicate that *Varroa* can have a disproportional effect on bees subjected to higher temperatures, hence parasitism and thermal stress may have synergic effects on survival and colony stability.

- On the other hand, the interaction between temperature and *Varroa destructor* has another high cost for the host, because it cause a decreasing

the number of cells and total protein in their hemolymph (Sadd and Schmid-Hempel, 2009), being a part of the explanation of the high energy cost of living in the interplay of these two factors (Luong *et al.,* 2017; Schmid *et al.,* 2008). I obtained an average of 1,210 cells/mm^3 in T_0 group at 32 °C; this is one fifth of the number described by Alaux *et al.,* (2010) and Szymaś and Jedruszuk (2003), but similar to that reported by Wilson-Rich *et al.,* (2008). Again, the number of mites affects to the bee, decreasing the number of cells significantly in bees parasitized with one or two mites as compared to the control. These results agree with earlier information that the number of free cells in hemolymph is affected negatively when the mite is present because of the healing response (Belaid and Doumandji, 2010; Koleoglu *et al.,* 2017; 2018; Wilson-Rich *et al.,* 2008); however another possible reason is the direct immune suppression effect described in parasitized bees (Ardia, 2012; Gregory *et al.,* 2005; Marringa *et al.,* 2014; Yang and Cox-Foster, 2005; 2007). If the warmer *versus* colder acclimation temperatures are compared, the number of cells in the T_0 group of bees at 38 °C was 16.7 % of the number at 32 °C, and in the T_1 group it was 26.4% of the number in the colder group; however this tendency was lost when the host was parasitized by two mites between acclimation temperatures. It is possible that when the bee is under a stressful temperature inside the hive, such as 38 °C, it releases cells into the hemolymph only when needed (fewest cells in T_0); such needs could be the presence of the Varroa mite. Alternatively, because the samples were taken just 60 min after parasitosis, the response is slower than under normal

temperature conditions and is just beginning, while the aggregation process is not yet happening ($T_0 < T_1 < T_2$). The other possibility is that free cells were present in the hemolymph and the aggregation process was inhibited by a direct effect of the mite (Kanbar and Engels, 2003).

- I found larger amounts of protein in bees than described by Wilson-Rich *et al.,* (2008) in nurse bees (30.12 ± 8.04 mg/mL *versus* less than 10 mg/mL); our results were closer to described by these authors for the pupae group. The amount of protein at 32 °C decreased from the value with no mites in groups with one mite (47.12 % of the T_0 value) and two mites (28.55% of the T_0 value). Although no other report has been found of decreasing protein in hemolymph, these results agree with previous reports that Varroa reduce the protein level in the tissues of the host (Bowen-Walker and Gunn, 2001). The Varroa mite is known to feed on fat body, and this tissue is the primary site of protein synthesis (Ramsey *et al.,* 2019). In the case of the warmer acclimatization temperature, the amount of protein in hemolymph was similar between uninfested and infested bees, but in all cases was lower than the normal values obtained in the T_0 group at 32 °C. No previous reports were found to compare between normal temperature and the effect on the total number of cells and protein in hemolymph when the ambient temperature is warmer, with or without mites on the bees.
- The individual survival rate decreased significantly between the groups of bees at 32 °C and at 38 °C: with no mites (57.5 % *vs.* 25 %); with one mite (42.5 % *vs.* 3.7 %); with two mites (40.0 % *vs.* 15 %). Our results agree with

Annoscia *et al.,* (2012), who remarked on the detrimental effects on survival when abiotic conditions change the relationship between the host and the parasite (Sadd and Schmid-Hempel, 2008). I have no explanation as to why the survival rate is higher in the T_2 group than in T_1 at 38 °C. As O'Connor and Bernhardt (2018) mentioned, changes in environmental factors such as temperature change the cost of parasitism in the host. We found that the survival rate is affected by temperature, but more strongly by the number of mites.

Conclusions

The interaction between temperature and number of mites is a trade-off between lifespan, metabolic rates, heat tolerance, individual survival and some hemolymphatic values of the host.

The energy cost for the bee is higher when it is parasitized, increasing when the individual has one or two mites, respectively. This means more energy expenditure related to the number of mites on each bee in both acclimation temperatures.

The heat tolerance of the bees is minor in cold acclimated than in warmer bees and is negatively effected by the number of mites.

The survival probability in non-parasitized bees is major between in cold and warmer acclimated bees and is reduced when the bees are parasitized with one or two mites, respectively.

In the hemolymph, the number of cells and the amount of total protein is reduced by the acclimation temperature, being major in cold acclimated than warmer bees and affected significantly by the number of Varroa.

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