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Warmer winters are associated with lower levels of the cryoprotectant glycerol, a slower decrease in vitellogenin expression and reduced virus infections in winter honeybees

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Abbreviations

IPCC - Intergovernmental Panel on Climate Change

G6PDH - glucose-6-phosphate dehydrogenase

NADPH - nicotinamide adenine dinucleotide phosphate

MW - mild winter

CW - cold winter

DWV - deformed wing virus

ABPV - acute bee paralysis virus

BQCV - black queen cell virus

Abstract

Within the context of climate change, winter temperatures at high latitudes are predicted to rise faster than summer temperatures. This phenomenon is expected to negatively affect the diapause performance and survival of insects, since they largely rely on low temperatures to lower their metabolism and preserve energy. However, some insects like honeybees, remain relatively active during the winter and elevate their metabolic rate to produce endothermic heat when temperatures drop. Warming winters are thus expected to improve overwintering performance of honeybees. In order to verify this hypothesis, for two consecutive years, we exposed honeybee colonies to either a mild or cold winter. We then monitored the influence of wintering conditions on several parameters of honeybee overwintering physiology, such as levels of the cryoprotectant glycerol, expression levels of immune and antioxidant genes, and genes encoding multifunctional proteins, including vitellogenin, which promotes bee longevity. Winter conditions had no effect on the expression of antioxidant genes, and genes related to immunity were not consistently affected. However, mild winters were consistently associated with a lower investment in glycerol synthesis and a higher expression of fat body genes, especially apidaecin and vitellogenin. Finally, while we found that viral loads generally decreased through the winter, this trend was more pronounced under mild winter conditions. In conclusion, and without considering how warming temperatures might affect other aspects of honeybee biology before overwintering, our data suggest that warming temperatures will likely benefit honeybee vitality by notably reducing their viral loads over the winter.

Keywords

Temperature, overwintering, metabolism, fat body, deformed wing virus, climate change

1. Introduction

Insects have a limited ability to regulate their internal body temperature hence, environmental temperature is one of the most important constraints on insect physiology, behaviour and survival (Angilletta, 2009; Chown and Nicolson, 2004). Climate change could thus profoundly affect insect population dynamics and distribution (Andrew et al., 2013; Lehmann et al., 2020).

Therefore, the influence of climate change on pollinators and their mutualistic interactions with plants have become a growing concern (Gilman et al., 2012; Hegland et al., 2009; Schweiger et al., 2010). Much of the research has focused on the consequence of climate warming on the potential shifts in geographic ranges (Dew et al., 2019; Kerr et al., 2015; Sirois-Delisle and Kerr, 2018) and the temporal mismatch between flowering time and insect pollinator emergence (see Gérard et al. (2020) for a review), which could result in declines of both plant and pollinator populations (Gilman et al., 2012). Direct effects of warming on pollinator physiology have also been investigated in the context of climate change (CaraDonna et al., 2018; Forrest et al., 2019; Holland and Bourke, 2015; Martinet et al., 2015; Sgolastra et al., 2011; Sgolastra et al., 2012), which could help to better understand temporal and spatial shifts in insect distribution as well as their performance and life-history responses (Scaven and Rafferty, 2013).

According to the report of the Intergovernmental Panel on Climate Change (IPCC, 2014), the increase in the global mean surface temperature by the end of the 21st century (2081-2100), relative to the period 1986–2005, will range from 1°C (5th to 95th percentile range: 0.3 to 1.7°C) for scenario RCP2.6, to 3.7°C (2.6 to 4.8°C) for scenario RCP8.5. Notably, temperatures are predicted to increase disproportionately during the winter in high latitudes (IPCC, 2014). An improvement in overwintering performance and survival is expected with the increasing winter temperatures. However, many insects generally rely on low temperatures to depress their metabolism and preserve their energy stores for the following spring (Brown et al., 2004; Williams et al., 2015). Therefore, higher temperatures associated with climate change are likely to have a negative impact on some species by increasing energy expenditure. As a consequence, overwintering insects with a tight energy budget will be especially vulnerable to warming (Hahn and Denlinger, 2011). In addition, freeze-thaw cycles, which are expected to increase with climate change, can reduce the freeze tolerance of insects (Bale and Hayward, 2010). Responses to higher pre-winter or winter temperatures have been studied in some pollinator species, and generally showed greater energy expenditure. However, while informative, most experiments were performed in artificial conditions with a constant temperature regime (Bosch and Kemp, 2003, 2004; Frund et al., 2013; Schenk et al., 2018; Sgolastra et al., 2010; Sgolastra et al., 2011), but see CaraDonna et al., 2018; Sgolastra et al., 2012). Therefore, less is known about the effects of natural wintering conditions, which include fluctuating temperatures and potential effects on insect biology (Colinet et al., 2015; Paaijmans et al., 2013).

Performing such an investigation is especially relevant for the western honeybee *Apis mellifera*, a social species of great economic importance due to the pollination services it provides to a wide

variety of crops (Breeze et al., 2014). The overwintering success of honeybees is critical for the pollination of early-blooming plants such as almonds. Furthermore, overwintering is known to be a highly sensitive period in the colony life cycle as indicated by the high winter losses reported in the United States (around 44%) (Seitz et al., 2015) and in Europe (from 2 to 32%) (Gray et al., 2020; Jacques et al., 2017). Contrary to most insect species, which go through a diapause stage during the winter, honeybees nest above the ground, which make them vulnerable to climatic conditions, and remain relatively active during the winter period although brood rearing is interrupted for most parts of the winter. When ambient temperatures drop below 10°C, bees cluster together for better thermoregulation (reduction of heat loss and endothermic heat production) (Heinrich, 1979, 1981; Stabentheiner et al., 2003). Consequently, the metabolic rate of the honeybee swarm remains stable when temperatures fluctuate between 10°C and 20°C, but when ambient temperatures drop below 10°C the honeybee swarm steeply increases its metabolic rate (Heinrich, 1981). In contrast to the expected effects on diapausing insects, warming winter temperatures are expected to reduce energy depletion and improve overwintering performance of the honeybee.

In order to verify this hypothesis and better understand the influence of winter climatic conditions on honeybees, for two consecutive years, we exposed colonies to mild or cold winters and tracked their physiology throughout the fall and winter seasons. Winter bees are characterized by physiological features that enable them to survive during the cold period, like an increase in nutrient storage in the fat body (Döke et al., 2015). The fat body, which plays an essential role in storing and releasing energy in response to energetic demands, is also involved in the secretion of several relevant molecules, such as antimicrobial peptides, vitellogenin and transferrin. For instance, vitellogenin, which can represent up to 40% of the total hemolymph protein fraction in worker bees, promotes longevity by acting as an antioxidant (Seehuus et al., 2006) and has an important role in immunity (Amdam et al., 2004; Salmela et al., 2015). Transferrin, which is also at high levels in winter bees (Erban et al., 2013), reduces oxidative stress and enhances survival from infections (Geiser and Winzerling, 2012; Kucharski and Maleszka, 2003). Temperature-induced changes in fat body nutrient storage and gene expression are therefore expected to affect bee vitality. In addition, ambient temperatures have notably been shown to have positive or negative effects on the immune functions of insects (Adamo and Lovett, 2011; Karl et al., 2011). We thus analyzed the expression levels of vitellogenin, transferrin and the antimicrobial peptides apidaecin, hymenoptaecin, as well as phenoloxidase, an enzyme involved in insect immunity through the melanization of foreign bodies, such as pathogens (González-Santoyo and Córdoba-Aguilar, 2011). The adaptive physiological response to cold was also assessed by measuring the production of glycerol (polyol cryoprotectants) (Clark and Worland, 2008), the enzyme activity of glucose-6-phosphate dehydrogenase (G6PDH) involved in the production of nicotinamide adenine dinucleotide phosphate (NADPH) for the synthesis of glycerol (Storey et al., 1991), and the expression levels of antioxidants (catalase and superoxide dismutase) (Joanisse and Storey, 1996; Wang et al., 2014). The expression level of *cyp4g11*

(cytochrome P450), likely involved in stress response (including low temperatures), was also assessed (Shi et al., 2013). Finally, we tested whether viral loads are affected by winter conditions, which could be the result of direct thermal effects on viruses (Kobayashi et al., 1981) and/or temperature-induced changes in host immune functions (Ferguson et al., 2018). For instance, it is generally assumed that insect hosts become more resistant to viral infection with increasing temperature (Frid and Myers, 2002; Kobayashi et al., 1981; Ribeiro and Pavan, 1994). We focused here on viruses that are amongst the most widespread and virulent for bees (deformed wing virus (DWV), acute bee paralysis virus (ABPV) and black queen cell virus (BQCV)), and that have been associated with the collapse of colonies (DWV and ABPV) (McMenamin and Genersch, 2015).

2. Materials and Methods

2.1. Experimental setup

During the fall season, twenty colonies consisting of ten-frames (mixture of *Apis mellifera ligustica* and *Apis mellifera mellifera*) with similar population size (around 20 000 bees) and resource storage (honey and pollen) were selected, after visual inspection, from a single apiary located near Salon-de-Provence, France (43° 38' 41.7"N, 5° 0' 55.5"E). These colonies were thus exposed to the same environmental resources and climatic conditions before and during the overwintering preparation. Because the parasite *Varroa destructor* can adversely affect the winter survival of honeybees (Dainat et al., 2012), colonies were treated against this mite at the end of summer (Apistan). Then, at the end of the fall, half of the colonies were randomly attributed to two groups and either moved to an apiary in Avignon (43° 54' 52.384"N, 4° 52' 42.884"E) (mild winter - MW) or in Theix (45° 42' 52.114"N, 3° 1' 40.385" E) (cold winter - CW). Both apiaries were located near a local weather station recording data since 1985. Previous recordings (from 2008 to 2012) indicated a temperature difference of around 5°C between the two apiaries during the fall/winter periods (October 1st to March 31).

To monitor the influence of climatic conditions on bee wintering physiology and viral loads, we sampled bees from each colony throughout the fall and winter. On October 22, 2013, colonies were translocated to their new sites (n=10 colonies per site) and ~100 bees per colony were sampled from the frames in 50 ml centrifuge tubes and placed in dry ice before storing them at -80°C. Bee sampling was also performed twice during the winter (December 12, 2013 and February 19, 2014) and once at the beginning of spring (March 27, 2014). To avoid any damaging effect of sampling on the winter cluster and therefore bee survival, sampling was performed when the air temperature was above 12°C (no visible winter cluster). The same experimental procedures were repeated the next year with twelve different colonies. On October 23, 2014, colonies were randomly selected and either moved to Avignon or Theix (n=6 colonies per site), then ~100 bees were collected, transported in dry ice and stored at -80°C. Further samplings were performed on December 19, 2014 and February 18, 2015 and finally March 31, 2015. All colonies survived through the winter except one, which died between the third and fourth sampling event at the Theix site (winter 2014/2015).

2.2. Winter physiology

The influence of winter climatic conditions on bee physiology was determined by measuring the amount of glycerol and G6PDH activity via enzymatic assays and the expression levels of immune genes (apidaecin, hymenoptaecin and phenoloxidase), genes involved in oxidative stress reduction (catalase, superoxide dismutase), and genes encoding multifunctional proteins (vitellogenin, transferrin, *cyp4g11*). Each physiological parameter was measured in both winters (2014 and 2015), except G6PDH activity, which was analysed only during the winter of 2015.

2.2.1. Enzymatic assays

Enzyme activities were assayed in abdomens devoid of the gut. All analyses were performed on 6 pools of 3 bees per colony and time point. Samples were homogenized at 4°C with a TissueLyser (Qiagen) (5 x 10 s at 30 Hz) in the lysis buffer (10 mM NaCl, 1% (w/v) Triton X-100, protease inhibitors (2 µg/ml antipain, leupeptin and pepstatin A, 25 units/ml aprotinin and 0.1 mg/ml soybean trypsin inhibitor), 40 mM sodium phosphate, pH 7.4) based on the weight of each pool (10% w/v extract) (Belzunces et al., 1988). After 10 min, the homogenization procedure was repeated a second time. The homogenate was centrifuged for 20 min at 15 000 g, and the supernatant was used to analyse G6PDH activity and the glycerol level. All procedures were performed at 4 °C.

Glucose-6-phosphate dehydrogenase

G6PDH is an enzyme of the pentose phosphate pathway, which contributes to the regeneration of reduced glutathione involved in the protection of cells against oxidative stress through the generation of NADPH. G6PDH, in the presence of the oxidized form of NADP+, catalyses the conversion of glucose-6-phosphate into 6-phosphogluconolactone and the reduced form of NADPH. G6PDH activity was determined by continuously following the formation of NADPH at 340 nm. The reaction medium contained 100 mM Tris-HCl buffer at pH 7.4, 10 mM MgCl2, 1 mM G-6-P, 0.5 mM NADP+ and 100 mM Tris-HCl pH 7.4 (see Renzy et al., 2016 for more details).

Glycerol

Glycerol is a major component of triglycerides. Under various physiological conditions, triglycerides are metabolized to release free fatty acids and glycerol. We used the Glycerol Colorimetric Assay Kit (Cayman chemical) for determining glycerol level in bee abdomens following the kit instructions. Glycerol was measured by a coupled enzymatic reaction system where glycerol is phosphorylated by glycerol kinase to produce adenosine-5'-diphosphate (ADP) and glycerol-3-phosphate. This latter is oxidized by glycerol phosphate oxidase to product dihydroxyacetone phosphate and hydrogen peroxide. The product of the redox reaction of H_2O_2 with 4-aminoantipyrine and N-ethyl-N-(3-sulfopropyl)-m-anisidine catalysed by peroxydase was followed at the absorbance of 540 nm.

2.2.2. Gene expression analysis

The expression level of genes was determined by quantitative RT-PCR on one and two pools of 30 bees per colony and time point for the winters 2014 and 2015, respectively. For the 2015 winter samples, each pool of 30 bee abdomens was divided in 3 for homogenization. The 10 abdomens were homogenized in 1 ml of Qiazol reagent (Qiagen) with a TissueLyser (Qiagen) (4 x 30 s at 30 Hz). The homogenates were incubated for 5 min at room temperature and after centrifugation (12,000 g for 30 s at 4° C), the 3 supernatants were pooled (167 µl each, giving 501 µl of supernatant). RNA extraction was then carried out as indicated in the RNeasy Plus Universal kit (Qiagen) with DNAse treatment. The concentration of RNA was determined with a NanoDrop spectrophotometer (Thermo Scientific). RNA solution was ten-fold diluted and cDNA synthesis was performed using 1,000 ng of RNA with the High capacity RNA to cDNA Kit (Applied Biosystems). After a ten-fold dilution of cDNA samples, the expression level of genes was assessed using a StepOne-Plus Real-Time PCR System (Applied Biosystems®) and the SYBR green detection method including the ROX passive reference dye. The reaction mixture consisted of 3 µl cDNA, 5 µl SYBR Green Master Mix (Applied Biosystems), 1 µl of forward primer (10 µmol) and 1 µl of reverse primer (10 µmol) of candidate gene. Primer sequences are shown in Table S1. A melting curve for the PCR products was performed at the end of each run to ascertain the reaction specificity. Quantification cycle (Cq) values were normalized to the geometric mean of the housekeeping genes actin and e*IF3-S8* using the comparative quantification method (delta Cq method).

2.3. Viral loads

Analyses of honeybee viral loads (DWV, BQCV and ABPV) were performed on the samples used for the quantification of gene expression levels (primers sequences in Tables S1). We used the same real-time PCR methods as those for the gene expression assays. However, for each virus, a standard curve using 10-fold serial dilutions of viral synthetic fragments of known concentration was used to calculate viral loads (Mondet et al., 2014). Positive and non-template controls were included on each plate. Viral load was then expressed as log-transformed equivalent copies of virus per bee. When the Cq value was above 35 for a given viral target, the sample was assigned as negative and given hypothetical Cq value of 36 for virus load analysis (Mondet et al., 2014).

2.4. Statistical analysis

All statistical analyses were performed using R version 3.5.1 (Team, 2020). Normality in the dataset at each site and time point was assessed via Shapiro-Wilk Tests. Differences in temperature between the two sites were assessed via Wilcoxon Rank Sum Tests. Differences in glycerol levels and G6PDH enzyme activity were assessed via mixed effect models using the hive as a random factor with the *lmer*

function of the *lme4* package. P-values for the coefficients of the models were obtained via Satterthwaite's degrees of freedom method using the *lmerTest* package (Kuznetsova et al., 2017). Changes in gene expression and viral loads in relation to winter condition (MW or CW) and time point were assessed via a two-way repeated measures ANOVA considering the hive as the subject. Post-hoc T-tests with a Bonferroni correction of the p-values were used to assess pairwise differences between winter conditions at each time point.

3. Results

3.1. Overwintering temperatures

Temperatures differed significantly between Theix and Avignon during the 2014 and 2015 winters, *i.e.* from October 2013 to March 2014 and from October 2014 to March 2015, respectively (Fig. S1). During the 2014 winter, the mean hourly temperature was 4.1 ± 4.9 °C and 9.4 ± 4.8 °C at Theix and Avignon, respectively (Wilcoxon Test: $p<0.001$). Similar temperatures were observed during the 2015 winter (Theix: 3.4 ± 5.2 °C and Avignon: 9.1 ± 4.8 °C; $p<0.001$). This difference is also evidenced by the number of hours above 10°C, temperatures above which bees do not form a winter cluster (Winter 2014: 521 and 1558 hours, Winter 2015: 319 and 1563 hours at Theix and Avignon, respectively; Figure 1).

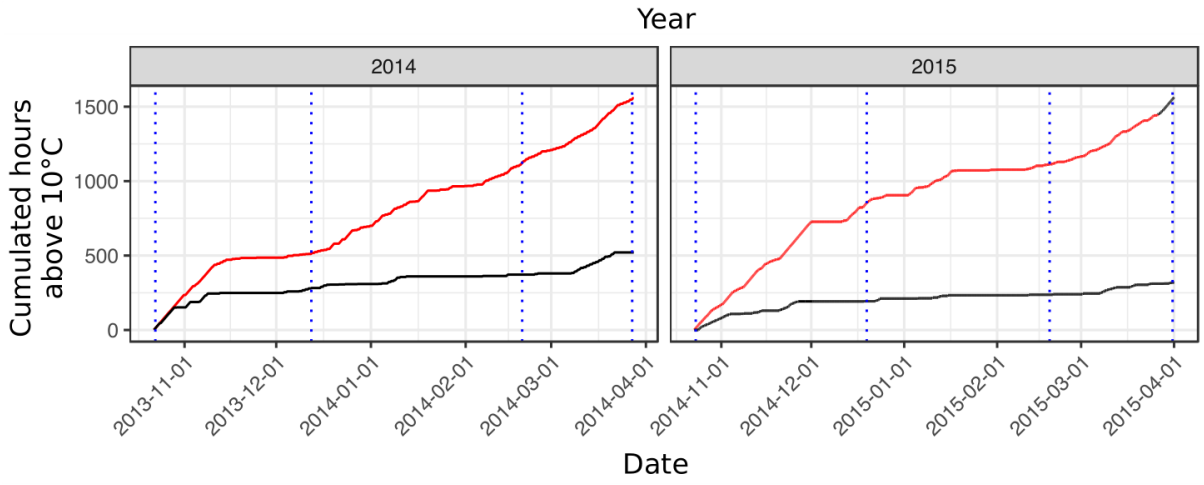

Figure 1. Cumulated number of hours above 10°C during the winters 2014 and 2015 in Avignon (red lines) and Theix (black lines). The dotted lines indicate the 4 sampling events at each winter.

3.2. Overwintering physiology

Glycerol levels showed that bees overwintering in Theix (cold winter – CW) were subjected to lower temperature stress than in Avignon (mild winter – MW). While glycerol levels did not differ immediately after hive relocation (T1), they increased during the CW and decreased during the MW

(Table 1 and Fig. 2A). This pattern was consistently observed both in 2014 and 2015. Similarly, the activity of the G6PDH enzyme did not differ between winters at T1, however a significant and stronger decline in activity was observed over the course of the CW as compared to the MW (Table 1 and Fig. 2B).

	Winter		**Time point**		**Winter x Time point**	
	F-value	P-value	F-value	P-value	F-value	P-value
2014						
Glycerol	1.1	0.3244	7.76	<0.001	5.07	0.0021
2015						
Glycerol	5.24	0.051	6.01	<0.001	37.37	<0.001
G6PDH	4.66	0.0628	15.34	<0.001	3.41	0.01816

Table 1. Mixed-effect model results of the influence of winter conditions and time on glycerol levels and G6PDH activity.

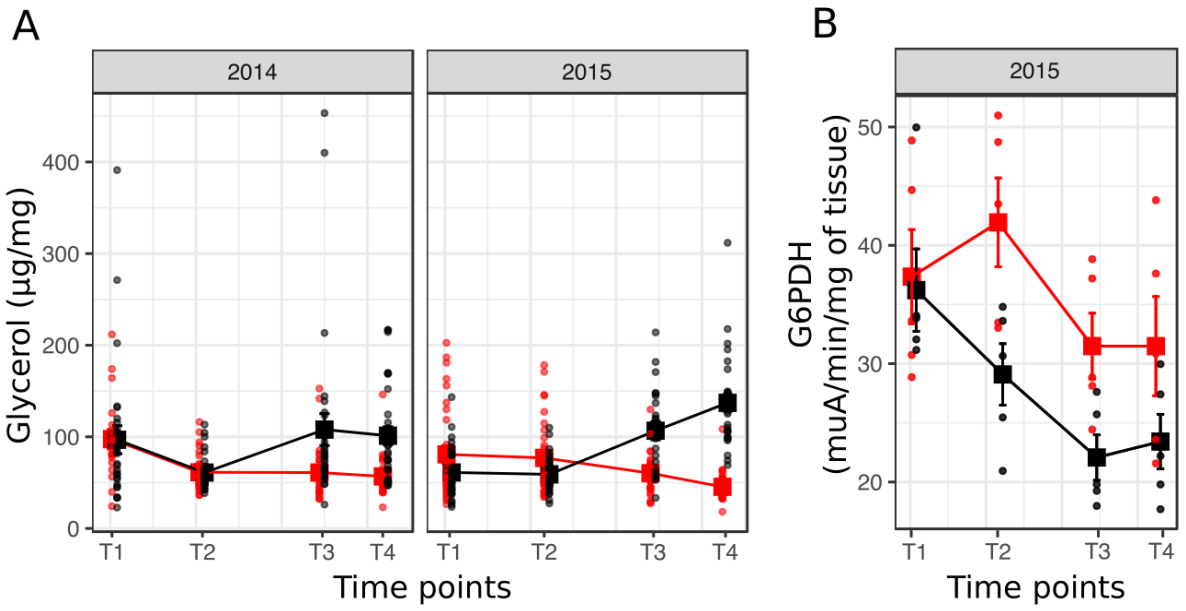

Figure 2. Amount of cryoprotectant glycerol (A) and G6PDH activity (B) in bee abdomens as a function of wintering conditions. Glycerol and G6PDH were analyzed throughout the CW (black) and MW (red) in pools of 3 bees (n= 6 pools per colony and time point; n=10 and 6 colonies per winter in 2014 and 2015, respectively). G6PDH activity was only measured in 2015. The average values (full square) and standard errors are shown. T1 to T4 indicate the 4 sampling events at each winter, and the x axis illustrates the number of days between sampling events.

The expression level of all measured genes changed over time during both winters, with the exception of catalase in 2015, which was at the limit of significance (see time points in Table 2, and Fig. 3 and 4). No change according to wintering conditions was found on the expression levels of catalase, superoxide dismutase, phenoloxidase and *cyp4g11* (Table 2). Furthermore, except for *cyp4g11*, which initially decreased and then increased by the end of winter, the expression patterns did not exhibit the

same trend over time across both years. In fact, a significant and consistent effect of the interaction of winter with time (*i.e.* both in 2014 and 2015) was found only for *cyp4g11*: at T1 its level did not differ between wintering conditions but bees from the CW tended to exhibit lower levels compared to the MW (especially in 2015) (Table 2 and Fig. 3).

Major changes were observed for apidaecin, hymenoptaecin, transferrin and vitellogenin, which all share expression in the fat body (Table 2 and Fig. 4). Their expression levels declined over both winters and were significantly affected by wintering conditions (with the exception of transferrin in 2014). At T1, the expression of these fat body genes did not differ but showed a general trend toward a stronger decline in expression under the CW conditions. An exception was found in 2015 for hymenoptaecin, which already exhibited a higher level at T1 in Avignon. Finally, apidaecin and vitellogenin, which showed differences in relation to sites and similar trends in both years, appear to be the most sensitive to wintering conditions (Table 2 and Fig. 4).

	Winter		**Time point**		**Winter x Time point**	
	F-value	P-value	F-value	P-value	F-value	P-value
2014						
catalase	1.083	0.312	**15.16**	**<0.00001**	**6.33**	**<0.001**
superoxide dismutase	2.855	0.108	**23.523**	**<0.00001**	2.462	0.0723
phenoloxidase	0.001	0.98	**12.95**	**<0.0001**	2.51	0.068
cyp4g11	4.28	0.053	**14.45**	**<0.00001**	**2.88**	**0.0442**
apideacin	**33.02**	**<0.0001**	**12.69**	**<0.00001**	2.26	0.0634
hymenoptaecin	**4.866**	**0.041**	**4.008**	**0.012**	0.531	0.663
transferrrin	4.539	0.472	**27.5**	**<0.00001**	0.192	0.902
vitellogenin	**6.046**	**0.024**	**30.272**	**<0.00001**	**8.257**	**<0.001**
2015						
catalase	2.461	0.151	2.721	0.0502	1.857	0.144
superoxide dismutase	1.704	0.224	**6.708**	**<0.001**	0.978	0.408
phenoloxidase	4.176	0.071	**3.745**	**0.0144**	1.92	0.1335
cyp4g11	5.11	0.0501	**9.224**	**<0.0001**	**3.196**	**0.0281**
apideacin	**10.06**	**0.011**	**30.844**	**<0.00001**	**9.975**	**<0.0001**
hymenoptaecin	**8.186**	**0.0187**	**7.794**	**<0.001**	**2.773**	**0.047**
transferrrin	**28.743**	**<0.001**	**27.838**	**<0.0001**	**8.843**	**<0.0001**
vitellogenin	**33.486**	**<0.001**	**17.11**	**<0.00001**	**12.63**	**<0.0001**

Table 2. Repeated Measures ANOVA results on the effects of winter conditions and time on the expression levels of genes.

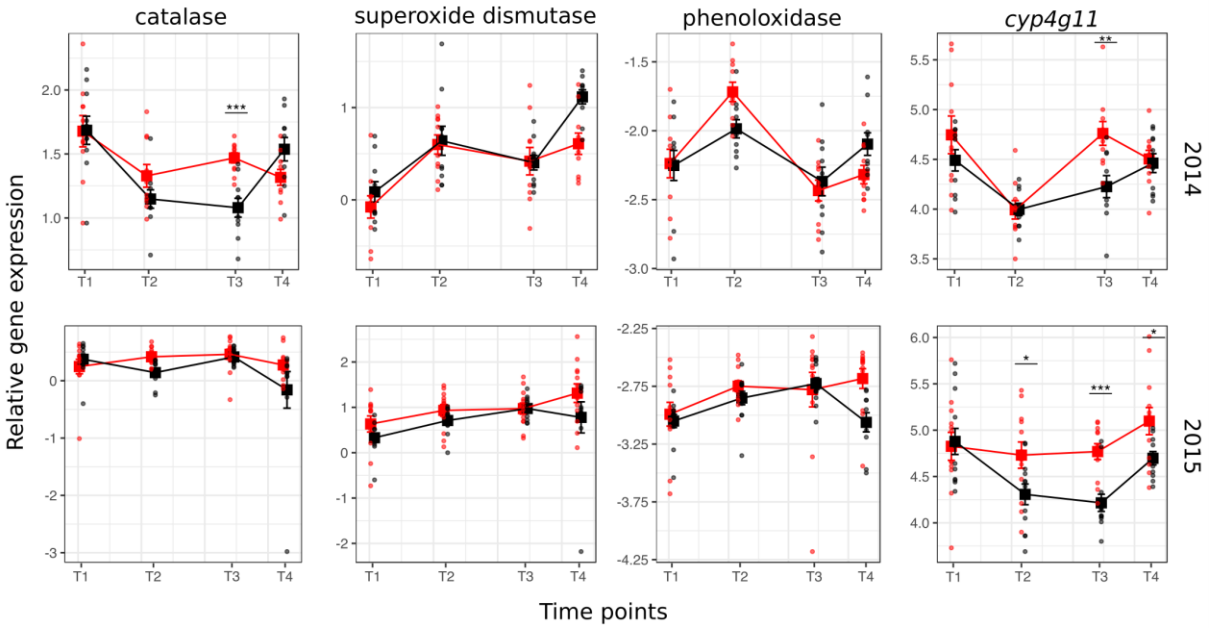

Figure 3. Expression levels of catalase, superoxide dismutase, phenoloxidase and *cyp4g11* as a function of wintering conditions. Gene expressions levels were determined throughout the CW (black) and MW (red) in pools of 30 bees (n= 1 and 2 pools per colony and time point in 2014 and 2015, respectively; n=10 and 6 colonies per winter in 2014 and 2015, respectively). The average values (full square) and standard errors are shown. Stars show significant differences of post hoc T-tests (*<0.05; **<0.01, ***<0.001, ****<0.0001). T1 to T4 indicate the 4 sampling events at each winter, and the x axis illustrates the number of days between sampling events.

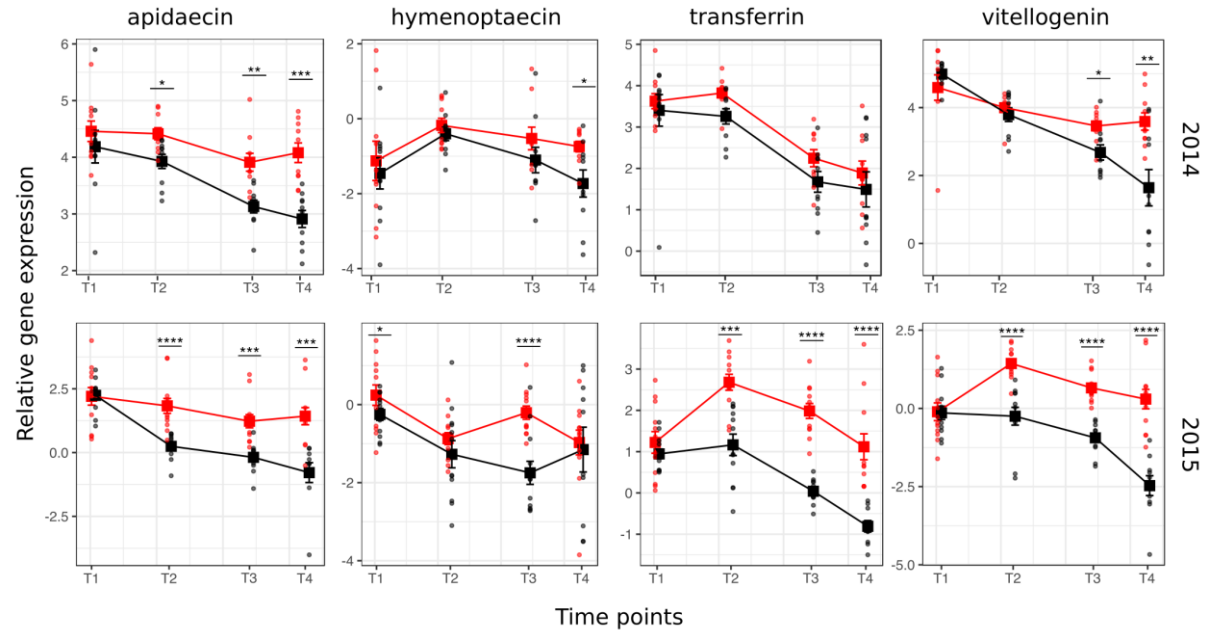

Figure 4. Expression levels of genes expressed in the fat body (apidaecin, hymenoptaecin, transferrin and vitellogenin) as a function of wintering conditions. Gene expression levels were determined throughout the CW (black) and MW (red) in pools of 30 bees (n= 1 and 2 pools per colony and time point in 2014 and 2015, respectively; n=10 and 6 colonies per winter in 2014 and 2015, respectively). The average values (full square) and standard errors are shown. Stars show significant differences of post hoc T-tests (*<0.05; **<0.01, ***<0.001, ****<0.0001). T1 to T4 indicate the 4 sampling events at each winter, and the x axis illustrates the number of days between sampling events.

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3.3. Overwintering viral loads

BQCV and DWV were detected in all bee samples. ABPV prevalence was also high (80% and 91% of colonies infected in 2014 and 2015, respectively), however at the end of the winter 2014 (T4), only 1 colony from the MW was found to be infected with ABPV as compared to 7 colonies in the CW (see
330 supplementary files - raw data).

ABPV, BQCV (excepted in 2015) and DWV loads exhibited significant changes over the course of winter (Table 3 and Fig. 5). Contrary to ABPV and DWV loads, which generally declined over time, an increase in BQCV loads was observed at the end of the winter. In addition, there was a significant influence of wintering conditions on viral loads (Table 3). The infection levels did not differ at the
335 beginning of the survey, but bees exposed to a MW became less infected than the bees experiencing a CW, with the exception of APBV in 2015 where no difference was found between the two winters (Table 3 and Fig. 5). The difference in viral loads between the two treatments was especially high for DWV.

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	Winter		**Time point**		**Winter x Time point**	
	F-value	P-value	F-value	F-value	P-value	F-value
2014						
ABPV	**9.04**	**<0.01**	**19.6**	**<0.0001**	0.953	0.429
BQCV	**12.75**	**<0.005**	1.24	0.304	1.815	0.155
DWV	**18.94**	**<0.001**	**3.764**	**0.0164**	1.736	0.1705
2015						
ABPV	0.48	0.489	**27.014**	**<0.0001**	0.087	0.967
BQCV	**84.36**	**<0.0001**	**48.07**	**<0.0001**	**15.26**	**<0.0001**
DWV	**36.42**	**<0.0001**	**69.97**	**<0.0001**	**3.54**	**0.018**

Table 3. Repeated Measures ANOVA results on the effect of winter conditions and time on virus levels.

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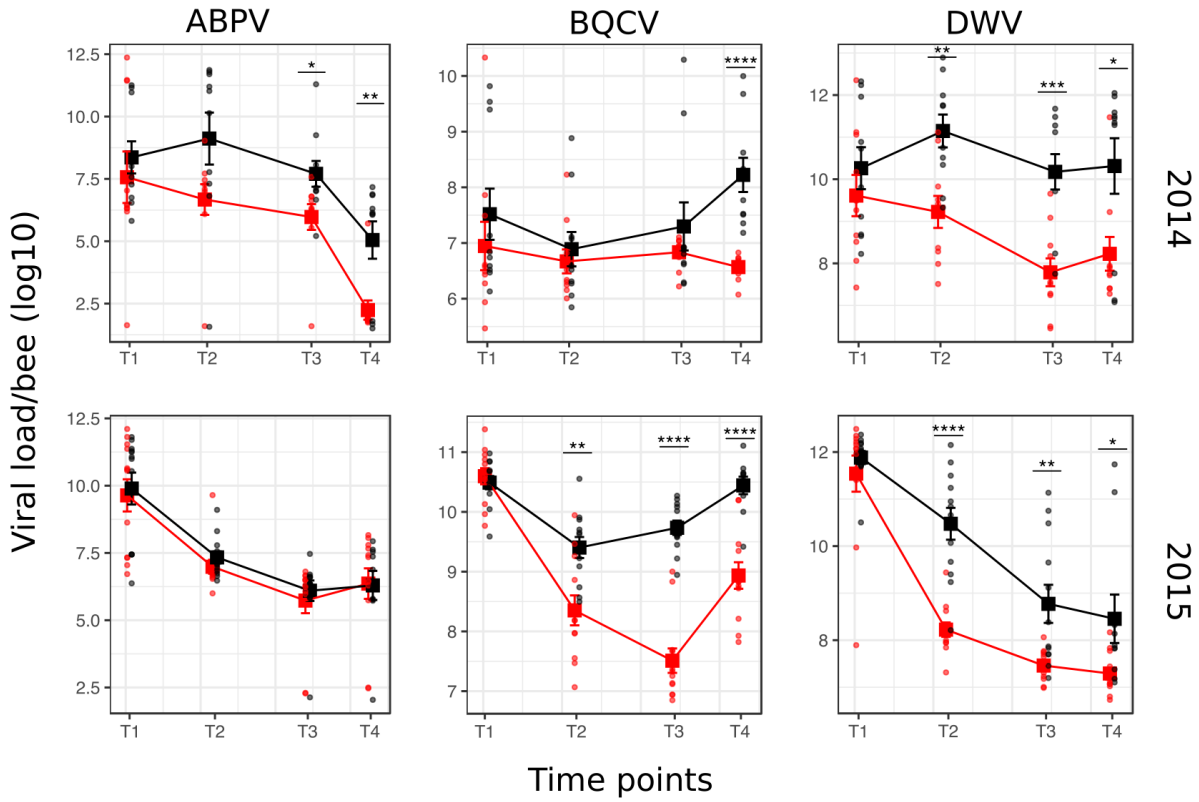

Figure 5. Viral loads as a function of wintering conditions. Virus loads were determined throughout the CW (black) and MW (red) in pools of 30 bees (n= 1 and 2 pools per colony and time point in 2014 and 2015, respectively; n=10 and 6 colonies per winter in 2014 and 2015, respectively). The average values (full square) and standard errors are shown. Stars show significant differences of post hoc T-tests (*<0.05; **<0.01, ***<0.001, ****<0.0001). T1 to T4 indicate the 4 sampling events at each winter, and the x axis illustrates the number of days between sampling events.

4. Discussion

In the current context of climate change, the goal of our experiments was to elucidate the influence of winter climatic conditions on honeybee physiology. By performing a two-year survey, we found that warmer winters were consistently associated with a lower investment in glycerol synthesis and more stable levels of apidaecin and vitellogenin over time. This higher vitellogenin level is indicative of better honeybee vitality under warmer winter conditions, which was further confirmed by the stronger decrease in virus infection.

To protect tissues from cold temperature and desiccation during overwintering, the most common strategy developed by insects is the synthesis of cryoprotectants, like glycerol, which prevent ice formation and help in reducing cell dehydration (Bale, 2002). An increase in glycerol has been notably observed in overwintering honeybees (Qin et al., 2019). Our data confirmed the accumulation of this cryoprotectant in bees exposed to the cold winter. In contrast, in the mild winter treatment, glycerol did not increase and remained stable both in 2014 and 2015. This shows that glycerol synthesis is temperature-dependant in honeybees and that mild winters might help bees to save energy since glycerol is released by lipolysis in adipose tissue (hydrolysis of lipid triglycerides into glycerol and

fatty acids). Glycerol can also be synthesized from the glycolytic pathway; 86% of the total carbon flow must be routed through the shunt between the pentose phosphate and glycolytic pathways to generate the required energy for glycerol synthesis (Storey and Storey, 1988). During the mild winter, we could therefore expect lower activity of G6PDH, which is the first enzyme of the pentose phosphate pathway. However, G6PDH was higher in the mild winter indicating that glycerol was mainly derived from lipolysis, but for which measurements of lipid levels would be required to confirm. The G6PDH enzyme is also involved in the generation of NADPH; thus, our results may indicate an increased protection against oxidative stress in the MW. The production of antioxidant defences has been shown to be an important mechanism protect insects against oxidative stress during overwintering (Joanisse and Storey, 1996; Sim and Denlinger, 2011). However, the hypothesis of a higher protection against oxidative stress does not seem supported by our study since the expression level of SOD and catalase did not differ between sites. An alternative explanation is the maintenance of a higher biosynthetic activity under mild winters since the products originating from the pentose phosphate pathway are used for fatty acid, nucleic acid and aromatic amino acid synthesis.

The expression pattern of *cyp4g11* is intriguing as it indicates a higher stress response (Shi et al., 2013) under the mild winter as compared to the cold winter (especially in 2015). By performing a functional characterization of CYP4G11, Calla et al. (2018) were able to demonstrate the multifunctional role of this protein. Their findings notably show the involvement of CYP4G11 in the production of cuticular hydrocarbons, which protect insects against desiccation (Krupp et al., 2020; Nelson and Lee, 2004). Thus, the higher *cyp4g11* in the MW might reflect an adaptation to cope with specific environmental conditions associated with higher desiccation stress, but this remains to be investigated.

Regarding immune functions, reduced expression of several immune genes has been reported in winter bees as compared to summer bees, suggesting a lower investment in immunocompetence in the winter due to either lower pathogenic pressures or decreased physiological activity (Steinmann et al., 2015). We could thus expect further reduction in immunity in bees exposed to cold winters as compared to bees exposed to mild winters. In addition, several studies reported temperature-induced changes in insect immune functions in either a positive or negative way (Adamo and Lovett, 2011; Ferguson et al., 2018; Karl et al., 2011; Medina et al., 2020). We did not find a clear link between wintering conditions and immune gene expression. Nonetheless apidaecin was consistently higher in bees exposed to mild winters. This suggests that if there is an influence of winter conditions on bee immunity it is not global but rather specific. Our result somehow confirms a longitudinal study that investigated the expression of several immune genes over several months in bees from 3 different apiary sites (Ricigliano et al., 2018): apidaecin expression was significantly influenced by the apiary site but not hymenoptaecin. The expression of apidaecin therefore seems to be particularly sensitive to local conditions.

Interestingly, the vitellogenin expression level declined over the winter but at a slower rate under the mild winter conditions in both years. Since vitellogenin is a marker of bee vitality and ageing (Alaux et al., 2017; Alaux et al., 2018; López-Uribe et al., 2020; Smart et al., 2016), we can assume that warmer winters might have a beneficial influence on bees. Transferrin was also higher in 2015 but not in 2014. In fact, the difference between wintering temperatures was less marked in 2014 as compared to 2015, which was reflected by reduced differences in apidaecin and vitellogenin expression levels between sites. It is not clear why warmer winters would slow down the decline of bee vitality, but one possible explanation is the slower depletion of energetic reserves from the fat body (as suggested by the lower glycerol synthesis). It is generally expected that warming winter temperatures elevate metabolic activity and thus deplete reserves in insects (Williams et al., 2003), however, in homeothermic organisms performing thermoregulation during cold winters, an increase in average temperature lowers their metabolic rates and therefore lessens their energetic stress (Williams et al., 2015). Like homoeothermic organisms, honeybees participate in endothermic heat production when temperatures drop by elevating their metabolic rates. Our data therefore suggest that warming winters will likely alleviate the depletion of fat body and thus sustain the expression of vitellogenin, critical to bee vitality. This is in accordance with a recent study, which showed that abdominal lipid contents were higher in honey bees that overwintered in controlled indoor storage facilities than in honeybees kept outdoors throughout the winter (Hopkins et al., 2021). Since the vitellogenin protein is used for jelly production and feed larvae (Amdam et al., 2003), the higher expression of vitellogenin under mild winters might also be explained by the presence of brood (for instance due the availability of some floral resources). Unfortunately, we did not thoroughly quantify brood production over the winter to avoid any perturbation of colonies, but we observed the presence of brood in both sites at the last sampling event (March). A general increase in vitellogenin expression would therefore be expected at this time, but this was not the case. Colony level parameters, such as brood presence, should however be included in future studies to better understand changes in bee physiological state over the winter.

Finally, a general tendency for a decrease in viral loads was observed over the winter, a decrease that was significantly more pronounced under mild winter conditions. This result is in agreement with the decline in viral loads previously described in honeybees exposed to high temperatures (Bordier et al., 2017; Dalmon et al., 2019; McMenamin et al., 2020). The increase in BQCV at the end of winter might be related to the start of brood production (as observed in both sites in March), since this virus, although common in adult bees, clinically affects larvae and pupae (Chen and Siede, 2007). The overall declining viral infection could result from either the death of highly infected bees (leaving the least infected bees within the colony), an increased resistance to viruses, or an inhibition of viral multiplication during overwintering. It is not clear why virus infection was better controlled under the mild winter conditions, but the reduced virus infection could be linked to a rather stable expression of apidaecin and/or vitellogenin, or to a higher expression of other immune mechanisms. For instance,

the protective effects of high temperature against virus have been attributed to the induction of heat-shock proteins (McMenamin et al., 2020). Additionally, the bee microbiome, which can affect disease susceptibility (Anderson and Ricigliano 2017), has been found to be sensitive to overwintering conditions (Maes et al., 2021).

By tracking bee physiology under two different winter regimes, we found that increasing winter temperatures will likely benefit honeybees by notably slowing down the decrease in vitellogenin expression and reducing their viral loads. We could therefore reasonably assume that warmer winter conditions will facilitate colony overwintering survival. However, we acknowledge that we only studied one mild winter and one cold winter site. While winter temperatures are the most likely key driver of the differences we observed in honeybee winter physiology, we cannot exclude the possibility that environmental differences other than temperature regime might have driven some of the physiological differences we observed between our sites. In addition, changes in environmental parameters occurring before the winter need also to be taken into account to better understand the potential effect of climate change on colony growth and survival. For instance, a mismatch between honeybee brood phenology and local environment might affect future colony growth (Nürnberger et al., 2019). In Austria a study found that warmer and drier weather conditions in the preceding year were associated with increased winter mortality (Switanek et al., 2017). A similar trend has been observed in the Netherlands where increased annual mean temperatures reduced winter survival (Yasrebi-de Kom et al., 2019). But in the northeastern United States, both too-cool and too-hot summers had adverse effects on colony winter survival (Calovi et al., 2021). In our experiments, colonies experienced the same climatic conditions before overwintering and solely the wintering conditions were changed. Furthermore, in the study of Switanek et al. (2017) warmer temperatures during the month of February were associated with decreased mortality, and in Belgium more frost days during the winter were associated with higher colony losses (Van Esch et al., 2020). Therefore, future studies on overwintering and climatic conditions should attempt to integrate preceding and actual wintering conditions, as well as winter duration, which will very likely be shortened given that climate change is predicted to induce early spring events (IPCC, 2014).

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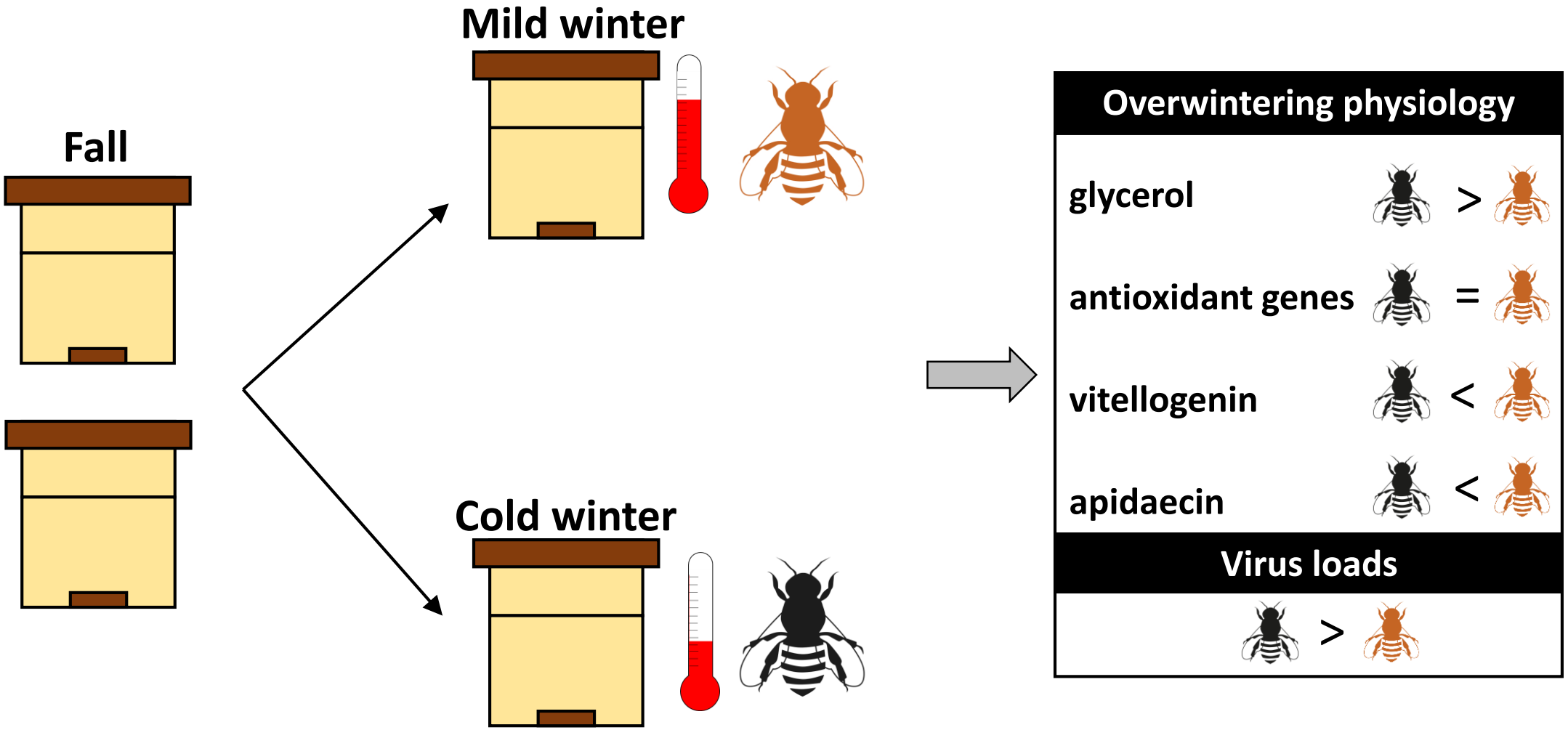
Fall
Mild winter
Cold winter
Overwintering physiology
glycerol >
antioxidant genes =
vitellogenin <
apidaecin <
Virus loads
>
Mild winter
Cold winter