Defensive strategies of *Apis mellifera mellifera* against the invasive alien species *Vespa velutina nigrithorax* in Bosland/Limburg

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Abstract

The dark European honeybee, *Apis mellifera mellifera*, an important pollinator and producer of bee products, has strongly declined in its native range within the last century. The invasive hornet *Vespa velutina nigrithorax* is an additional challenge for the bees conservation, as its presence reduces foraging activity and negatively impacts winter survival of honeybee colonies. This study aimed to assess the defensive behaviour of dark European honeybee colonies native in Limburg, Belgium, towards the invasive hornet. Honeybee colonies were exposed to a dead *V. v. nigrithorax* along with two control objects for shape and odour. In addition, the study explored a possible association between defensive behaviour against the hornet and gentleness towards the beekeeper, a trait actively selected for by beekeepers. Results showed responses on the hornet varied among colonies, suggesting potential for selection. This study did not reveal a significant association between defensive behaviour towards the hornet and gentleness towards beekeepers. However, the possibility of a negative correlation cannot be entirely ruled out. A survey indicated that gentleness remains a valued trait among beekeepers. Further research is needed to elucidate the relationship between defensive behaviour against *V. velutina* and gentleness in dark European honeybees to develop an effective breeding strategy.

Abstract

De zwarte bij, *Apis mellifera mellifera*, belangrijk als bestuiver en voor de productie van bijenproducten, is de laatste eeuw sterk afgenomen binnen zijn oorspronkelijke verspreidingsgebied. De invasieve hoornaar *Vespa velutina nigrithorax* vormt een aanvullende uitdaging voor de conservatie van de zwarte bij, aangezien de aanwezigheid ervan de foerageeractiviteit vermindert en de winteroverleving van honingbijenkolonies negatief beïnvloedt. Deze studie had als doel het verdedigingsgedrag van de in Limburg, België, inheemse zwarte bijenkolonies te beoordelen op hun reactie op de invasieve hoornaar. Bijenkorven werden hiervoor blootgesteld aan een dode *V. v. nigrithorax*, naast twee controleobjecten voor vorm en geur. Ook werd gekeken naar een mogelijke associatie tussen het verdedigingsgedrag tegen de hoornaar en zachtaardigheid naar de imker toe, een eigenschap waarop imkers actief selecteren. De gevonden variatie in reactie op de hoornaar tussen kolonies suggereert potentieel voor selectie voor weerbaarheid. Geen significante associatie tussen de reactie op de hoornaar en zachtaardigheid is gevonden, hoewel een negatieve correlatie niet kan worden uitgesloten. Een afgenomen enquête geeft aan dat zachtaardigheid voor de imker van belang blijft. Verder onderzoek naar een mogelijke link tussen het defensief gedrag van de zwarte bij tegen *V. velutina* en zachtaardigheid is aangeraden voor de ontwikkeling van een effectief kweekprogramma.

Introduction

Dark European honeybee

The western honeybee, *Apis mellifera*, is a social insect from the family Apidae within the order Hymenoptera. The species is native to Africa, Europe and Western Asia but is kept globally as an important producent of bee products and pollinator of crops MORITZ et al. (2005); GARIBALDI et al. (2017). *Apis mellifera mellifera*, commonly named the dark European honeybee, is a subspecies of the western honeybee. It occurs from France and the British Isles in western Europe to the region west of the Ural Mountains in northeastern Europe, and to southern Scandinavia in northern Europe DE LA RUA et al. (2009); ILYASOV et al. (2020). The loss of suitable nesting and foraging habitats, use of pesticides, introduction of pathogens and the import of other subspecies—resulting in hybridisation or complete replacement of *A. m. mellifera*—have led to a strong decline of *A. m. mellifera* throughout Europe REINSCH et al. (1991); DE LA RUA et al. (2009); MEIXNER et al. (2013); PASHTE & PATIL (2018). At present, multiple efforts exist to conserve genetic diversity and adaptability of the dark European honeybee, including in Limburg, Belgium DE LA RUA et al. (2009); BOSLAB (2021-2023).

Asian hornet

Particularly in the western part of its native range, the establishment of an invasive hornet species can pose an additional challenge to the survival of dark European honeybee colonies DE LA RUA et al. (2009); VILLEMANT et al. (2011); ARCA et al. (2014); REQUIER et al. (2019). *Vespa velutina*, commonly named the Asian hornet, is a social insect from the family Vespidae within the order Hymenoptera It is native to Asia from Afghanistan and Indochina to eastern China and Indonesia, where it is known to be a specialised predator of honeybees CARPENTER & KOJIMA (1997); TAN et al. (2007). The subspecies *Vespa velutina nigrithorax* was first observed in Europe in 2005 in Lot-et-Garonne, France, where it was presumably present since 2004 or even earlier HAXAIRE et al. (2006). The European population may be the result of a single introduction event with a single or very few queens from an area between the Chinese regions Zhejiang and Jiangsu, at the east coast of China, presumably imported in France by ship along with pottery VILLEMANT et al. (2006); ARCA et al. (2015). It successfully established itself after introduction and is, despite control measures, rapidly spreading across Europe, causing ecological, economic and to a lesser extent human life losses MONCEAU et al. (2014a); ROBINET et al. (2017); SCHOONVAERE et al. (2020); COURTIOUX (2021). In 2016, the species was included in the list of invasive alien species of Union concern of the European Union THE EUROPEAN COMMISION (2016). Economic impact of the Asian hornet is the highest on apiculture, by increasing honeybee colony losses and reducing productivity, and on agriculture, by causing damage to fruit crops and reducing the amount of pollinators MONCEAU et al. (2018); LAURINO et al. (2020); SCHOONVAERE et al. (2020); REQUIER et al. (2023); LUEJE et al. (2024). The first nest in Belgium was found in 2016 in Hainaut, and the species is known to be present in Limburg since 2018 SCHOONVAERE et al. (2020).

Predator-prey behaviour

Although observations in France show *V. v. nigrithorax* to be a generalist predator, social hymenopterans and especially *A. mellifera* appeared to be the preferential prey PERRARD et al. (2013); ROME et al. (2021). Honeybees consistently serve as an important and in general largest source of protein for the hornet colony MONCEAU et al. (2014a); REQUIER et al. (2019);

Rome et al. (2021). The Asian hornet shows specialized behaviour targeted towards honeybees, called bee-hawking, where it hovers 10 – 40 cm in front of the hive entrance to catch a bee. The hornet mostly focusses on returning foragers loaded with pollen, which may be slower and therefore easier to catch Tan et al. (2007); Tan et al. (2012); Monceau et al. (2013); Arca et al. (2014); Bunker (2019); Laurino et al. (2020). The number of the dark European honeybee workers engaging in foraging activity decreases with the number of hornets present in front of the hive, but also in time from early summer to October, when the predation pressure from the Asian hornet increases Monceau et al. (2013); Monceau et al. (2018); Rome et al. (2021). A reduction in foraging behaviour consequently leads to a decrease in the import of resources such as pollen and may increase the consumption of stored resources due to a higher number of adult bees remaining within the hive Reqiuer et al. (2019). *V. velutina* may enter weakened beehives to take brood, pollen and honey but most bee colonies unable to cope with the predation pressure die out later in winter due to depopulation and lack of resources Arca et al. (2014); Monceau et al. (2014b); Requier et al. (2019); Laurino et al. (2020); Schoonvaere et al. (2020).

The Asian honeybee, *Apis cerana*, which occurs in the native range of *V. velutina*, has developed behaviour adapted to cope with this hornet predation, including bee-carpet formation, balling, shimmering, and increasing flight speed when approaching the hive Tan et al. (2007); Sugahara & Sakamoto (2009); Tan et al. (2012). In the presence of hunting hornets, a bee-carpet can be formed at the hive entrance by numerous bees clinging together, which prevents lone bees to be picked off the hive by a hornet and may engulf hornets that come in contact with the bees Tan et al. (2007); Arca et al. (2014). Defending honeybees can kill a hornet through balling. This behaviour involves engulfing the hornet in a compact ball of bees in which an increase in temperature and CO_2 levels, combined with stinging, causes the death of the hornet Sugahara & Sakamoto (2009); Arca et al. (2014); Gu et al. (2021). Bee-carpet formation and balling behaviour also occur in *A. mellifera* though with lower efficiency compared to *A. cerana*. *A. mellifera* recruits fewer bees for both behaviours and a lower temperature is reached during balling, which may be sufficient under the mild predation pressure of *Vespa crabro* occurring in its natural range Ken et al. (2005); Tan et al. (2007); Baracchi et al. (2010). When both *A. mellifera* and *A. cerana* are present in its natural range, *V. velutina* prefers hunting the introduced western honeybee to reach a higher success rate Tan et al. (2007).

Defensive response of the dark European honeybee

Apparently the number of defending bees in front of the hive are related to the number of hornets present and the time of year when bee carpet formation is studied in *A. m. mellifera* Monceau et al. (2018). The dark European honeybee is able to kill *V. velutina* foragers by balling, but this behaviour is only known to occur when the hornet gets into the hive or the bee-carpet. It does not occur regularly enough to limit predation pressure Monceau et al. (2018). Papachristoforou et al. (2007) found that another subspecies of *A. mellifera*, *Apis mellifera cypria*, asphyxiates the hornet *Vespa orientalis* in its natural range by actively blocking the movement of tegites at its abdomen during balling. Whether this behaviour also occurs in *A. m. mellifera* is unknown. Tracking of a hornet in front of the beehive by a dark European honeybee worker can occur, and this behaviour occurs in varying amounts between colonies Monceau et al. (2018).

Variation in defensive behaviour as a potential for selection

Within the species *A. mellifera*, differences in defensive behaviour are found between subspecies and within a subspecies, including *A. m mellifera* RUTTENER (1988); KOLMES & FERGUSSON-KOLMES (1989); KASTBERGER et al. (2009); BARACCHI et al. (2010); KANDEMIR et al. (2012); UZUNOV et al. (2014); GUICHARD et al. (2020). Defensive behaviour of the dark European honeybee is largely genetically determined UZUNOV et al. (2014); GUICHARD et al. (2020); GUICHARD et al. (2021). As suggested by MONCEAU et al. (2018), heritable variation in defensive behaviour among dark European honeybee colonies could provide a selection potential for colonies that can more effectively defend against the Asian hornet.

The aim of this study was to assess the defensive behaviour of *Apis mellifera mellifera* colonies native in Limburg, Belgium, against the invasive *Vespa velutina nigrithorax*. A potential variability in colony responses to the invasive Asian hornet could provide a basis for developing a breeding program aimed at enhancing the defensive capabilities of the dark European honeybee if colonies with strong defensive responses are identified.

Possible link between defensibility and gentleness

Beekeepers apply intensive selection procedures on honeybees to promote gentleness towards humans. This, however, may negatively influence their ability to defend themselves against natural enemies UZUNOV et al. (2014). Although there are also selective breeding efforts on *A. m. mellifera*, this subspecies has been less subject to coordinated, long-lasting intensive artificial selection compared to other subspecies such as *Apis mellifera carnica* and *Apis mellifera ligustica* UZUNOV et al. (2014). Conversely, gentleness is also an important trait to consider, as beekeepers may import other honeybee varieties if they deem the native honeybee less favourable. The negative reputation of the dark European honeybee as an aggressive subspecies has contributed to the decline of this subspecies within the last century REINSCH et al. (1991); DE LA RUA et al. (2009); UZUNOV et al. (2014). In the dark European honeybee, defensive behaviour has been found to be associated with calmness during beehive inspection GUICHARD et al. (2020). Therefore, it is reasonable to assume that a link between defensive behaviour against the Asian hornet and gentleness may exist.

Additionally, this study aimed to investigated a whether there is a correlation between the defensive behaviour of *Apis mellifera mellifera* against *Vespa velutina nigrithorax* and its gentleness towards beekeepers, as gentleness may be considered an important trait by beekeepers.

Exploratory survey

In addition, an exploratory survey was conducted among beekeepers using a structured questionnaire. Most questions from the survey extended beyond the scope of this study and align with broader research themes. However, a specific section of the survey focused on the invasive Asian hornet and beekeepers' attitudes towards honeybee gentleness, inter alia in the context of this invasive hornet. This may provide insights into the potential conflict between selecting for gentleness and effective defensive capabilities against *V. v. nigrithorax*.

Methodology

Study area

The experiment was carried out on 44 *Apis mellifera mellifera* colonies located in Bosland National Park in Limburg, Belgium. These colonies were situated across five sites with similar environmental conditions within the national park. The colonies used in this study are part of the Bosland's Zwarte Bij Project which aims to reintroduce a stable population of the locally disappeared native dark European honeybee BosLAB (2021-2023).

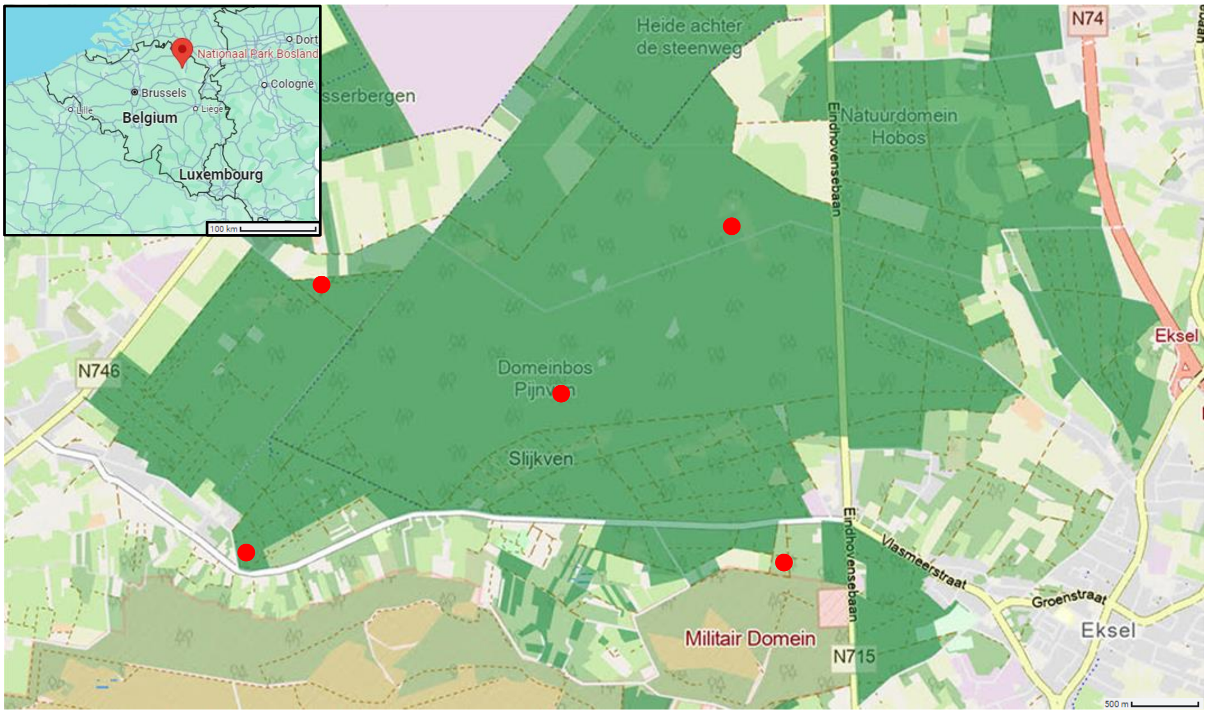

Figure 1: Location of the 5 study sites within Bosland National Park in Limburg, Belgium. The sites are marked with a red dot.

Study design

To assess the defensive behaviour of *Apis mellifera mellifera* colonies in Limburg against the invasive *Vespa velutina nigrithorax*, colonies were exposed to an Asian hornet to simulate a predatory threat. The colonies' responses were evaluated using a predefined scale. The data were then analysed for variation and compared with reactions to control objects. Additionally, the colonies were also rated for gentleness, which allowed testing for a possible association between the defensive response to the hornet and gentleness towards beekeepers.

An Asian hornet (*Vespa velutina nigrithorax*) was captured in front of a beehive for use during the experiment. In addition to the hornet, a piece of wood and a rod with queen bee pheromone 9-ODA were used as controls to account for shape and odour, respectively.

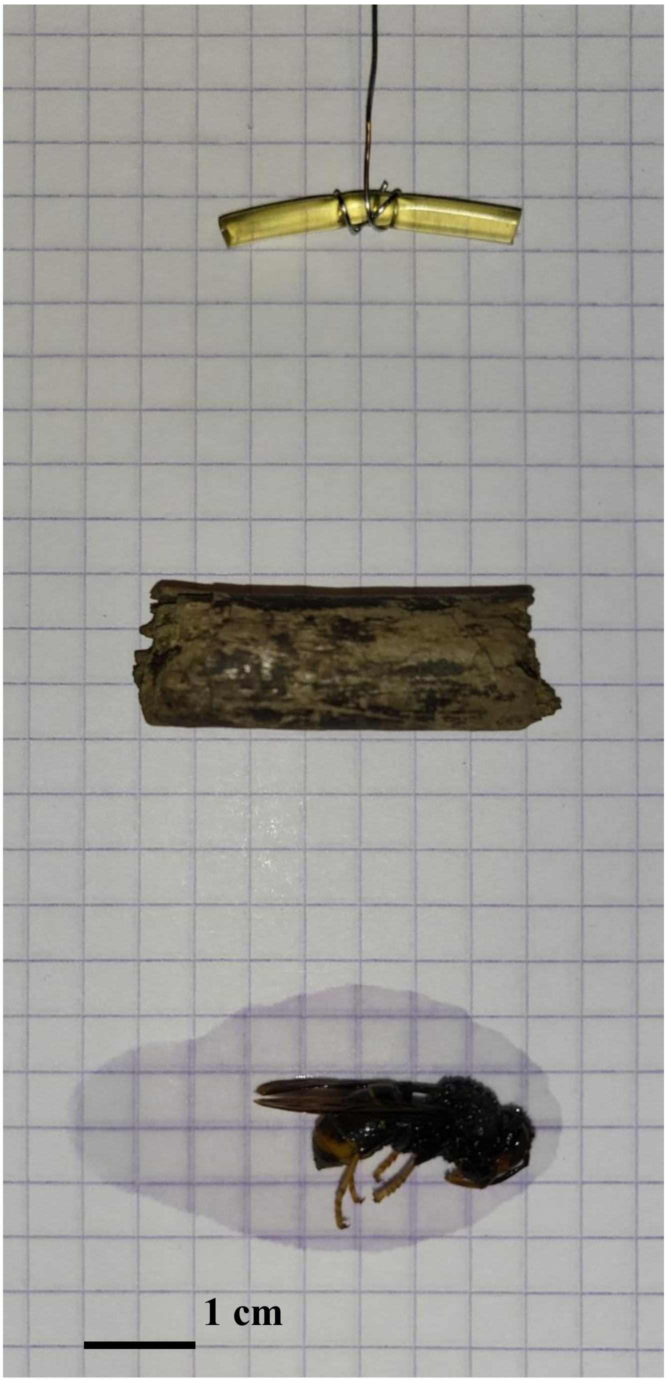

Figure 2: Test objects used during the experiment. From top to bottom: rod with queen bee pheromone (9-ODA), piece of wood, Asian hornet (Vespa velutina nigrithorax).

Experimental set-up

The experiment was conducted on 1 October, 2023. The weather conditions on the day of the experiment were sunny and dry, with a maximum temperature of 25°C. The hornet was caught two days in advance and preserved at a temperature of -20°C until the experiment was conducted.

To collect the data, a beehive was opened and the gentleness of a colony was rated on a scale from 0 to 3 depending on their reaction. A value of 0 is equivalent to an aggressive response, corresponding to a high number of bees flying towards a person opening the beehive and subsequently a high frequency of stinging occurs. A value of 1 is equivalent to a moderately aggressive response, corresponding to a modest number of bees flying around someone who opens their beehive and multiple stings. A value of 2 is equivalent to a moderately gentle response, corresponding to a few bees flying around someone who opens their beehive and 1 or 0 stings. A value of 3 is equivalent to a gentle response, corresponding to the bees remaining calm when someone opens their beehive and no stinging (see appendix 1 & appendix 2).

Subsequently, the response towards the test objects i.e. rod with queen bee pheromone (9-ODA), piece of wood, Asian hornet was rated by holding them simultaneously 2 decimetres above the opened beehive using an iron wire (Figure 3). Each object was scored on a scale from 0 to 3 based on the number of bees attracted to it. A score of 0 indicated no interest, corresponding to no bees on the object. A score of 1 indicated limited interest, corresponding to one bee on the object. A score of 2 indicated moderate interest, corresponding to two or three bees on the object. A score of 3 indicated strong interest, corresponding to more than three bees on the object (see appendix 1 & appendix 2). Additionally, it was noted whether the bees were merely inspecting or actively attacking the hornet, the latter behaviour involves biting and attempts to sting or pull the hornet away from the hive.

At the southeasternmost site, an extra test was performed in front of the hive entrance. After closing the beehive, the test objects were positioned 2 decimetres in front of the entrance, and the sampling procedure was repeated.

Additional information noted included whether a colony occupied a double-stacked beehive, and whether the location featured beehives that were completely shaded, partially shaded or exposed to full sunlight. The maternal line of each colony was known, and this information could be used to check for associations with the colony's response to the hornet.

Figure 3: Sampling set-up to assess bee colony response on different test objects. With iron wires, the objects are hold 2 decimetres above an opened beehive. Iron wires are indicated with a red arrow.

Additional data gathering

A similar dataset from August 2022 was also made available for analysis. This dataset includes tests at 52 *A. m. mellifera* colonies across two locations in Averbode and Langdorp in Flemish Brabant, Belgium. The procedure was consistent with those conducted in 2023 in Bosland National Park, except for the part where objects were always held at the hive entrance instead of above the open hive. The maternal line of each colony and the degree of shading was also known (see appendix 2). Additionally, the number of occupied frames in each beehive was documented for October and February, allowing for the determination of decline during winter through comparison. Active predation by *V. v. nigrithorax* was observed at some of the colonies.

Survey

A survey in the form of a structured questionnaire has been conducted during a symposium on the Asian hornet in February 2024 in Limburg and was distributed by e-mail. It examines demographic information, experience, geographical distribution, the beekeepers' purpose—commercial, secondary occupation or hobby—of beekeeping activities, types of honeybees used, and awareness of *Apis mellifera mellifera* being the local honeybee subspecies. It further addresses the importance of local adaptation, experiences with winter bee mortality, impact of the invasive *Vespa velutina nigrithorax* and seeks to gain insight in beekeepers' attitudes towards the gentleness of bees. Questions addressed in the survey are attached in appendix 3.

Statistical analysis

Assessing sample independence

Data was collected per study site. Kruskal-Wallis tests were used to assess differences between locations for both the data of 2022 and 2023. This was necessary to confirm that the data could be treated as independent samples, which was essential for the validity of subsequent analyses.

Analysis of honeybee responses

Non-parametric tests were chosen due to the non-normal distribution of the data. However, complete independence of observations could not be fully guaranteed, as the test objects were presented simultaneously to a hive during the experiment. Therefore, the results should not be interpreted without caution.

Kendall's tau and Spearman's rho were used on the 2022 and 2023 datasets, to test for correlations between: 1) response to the hornet and gentleness, 2) response to the hornet and response to the piece of wood, and 3) response to the hornet and response to bee pheromones. Additionally, in the 2022 data, an correlation between response to the hornet and colony decline during winter was examined. Wilcoxon rank sum tests were used to check if the response towards the hornet scored significantly higher than the response towards the piece of wood and rod containing bee pheromone.

Kruskal-Wallis tests were conducted on the 2022 dataset to asses associations between: 1) response to the hornet and maternal lineage, 2) gentleness and maternal lineage, 3) response to the hornet and surviving winter, and 4) response to the hornet and observed hornet predation. In this dataset, sun exposure was not tested due all beehives being partially shaded.

Kruskal-Wallis tests were conducted on the 2023 dataset to asses an association between: 1) response to the hornet and maternal lineage, 2) gentleness and maternal lineage, 3) response to the hornet and sunlight exposure, and 4) response to the hornet and occupying a double-stacked beehive.

Analysis of the survey dataset

Multiple analytical tests were conducted to examine potential associations between the responses of the exploratory survey.

Logistic regression analyses were used to test for an association between: 1) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and attributed value to colony gentleness, 2) believing it is important to work with a honeybee variety that is very well adapted to local weather conditions and attributed value to colony gentleness, 3) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and age, 4) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and years of beekeeping experience, 5) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and the purpose of beekeeping activities, 6) having indicated they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper intervention and age, 7) having indicated they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper intervention and years of beekeeping experience, and 8) having indicated they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper intervention and the purpose of beekeeping activities.

A McNemar test was used to check whether there was a significant difference between willingness to work with bee colonies that are better able to defend against *V. v. nigrithorax* and willingness to work with such bee colonies if they also exhibited aggression towards the beekeeper.

Chi-square tests were used to test for an association between: 1) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and having indicated being negatively affected by *V. v. nigrithorax*, 2) having indicated they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper intervention and having indicated being negatively affected by *V. v. nigrithorax*, 3) reporting having experienced colony losses last winter and having indicated being negatively affected by *V. v. nigrithorax*, 4) having indicated they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper intervention and willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax*, 5) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and reporting having experienced colony losses last winter.

Due to expected frequencies below 5, which did not allow for a reliable chi-square test, Fisher's exact tests were used to test for an association between: 1) willingness to work with bee colonies that are better able to defend against *V. v. nigrithorax* and having indicated being negatively affected by *V. v. nigrithorax*, 2) having indicated being negatively affected by *V. v. nigrithorax* and postal code, 3) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and the honeybee varieties a beekeeper works with, 4) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax* and believing it is important to work with a honeybee variety that is very well adapted to local weather conditions, 5) having indicated they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper intervention and the honeybee varieties a beekeeper works with, and 6) attributed value to colony gentleness and the honeybee varieties a beekeeper works with. The test for an association between attributed value to colony gentleness and the honeybee varieties a beekeeper works with is performed an additional time only involving answers of beekeepers who work exclusively with only one variety of honeybee.

A subset of the survey responses was created, only including the responses from beekeepers who reported experiencing colony losses last winter. Because the expected frequencies were below 5 which makes the chi-square test unreliable, the Fisher's exact was used on this subset to test for an association between indicating *V. v. nigrithorax* as a cause of colony losses and: 1) willingness to work with aggressive bee colonies that are better able to defend against *V. v. nigrithorax*, and 2) indicating they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper.

Spearman rank correlation tests were used to test for an association between the value attributed to colony gentleness and: 1) age, 2) years of beekeeping experience, and 3) the purpose of beekeeping activities.

Statistical software

All statistical analyses were conducted using RStudio, with R version 4.4.0. Statistical significance was assessed at $\alpha = 0{,}05$ level.

Results

Observational data

In both the datasets of 2022 and 2023, responses to the presented hornet varied among colonies, ranging from no response to strong interest involving multiple bees engaging with the hornet. No balling behaviour has been observed during the study.

Influence of environmental factors on honeybee responses

No significant impact of location on the response to the hornet was found for the data of 2022 (Kruskal-Wallis test: $\chi^2 = 1{,}0812$; df = 1; $P = 0{,}2984$) or the data of 2023 (Kruskal-Wallis test: $\chi^2 = 5{,}8491$; df = 4; $P = 0{,}2107$).

Table 1: Statistical tests evaluating the relationship between variables and the response of the dark European honeybee to the Asian hornet. Significant outcomes are highlighted in bold.

		Dataset 2022	Dataset 2023
Variable	**Statistical test**	***P*-value**	***P*-value**
Association with environmental factors			
Study site	Kruskal-Wallis	0,2984	0,2107
Sunlight exposure	Kruskal-Wallis		0,06192
Occupation of a double-stacked hive	Kruskal-Wallis		0,3255
Correlation with response to control objects			
Wood	Kendall's tau	0,9511	0,7069
Wood	Spearman's rho	0,9519	0,7116
Bee pheromone	Kendall's tau	0,3237	0,6869
Bee pheromone	Spearman's rho	0,3285	0,6971
Association with maternal lineage			
Maternal lineage	Kruskal-Wallis	0,6300	**0,02029**
Correlation with gentleness			
Gentleness	Kendall's tau	0,09787	0,05184
Gentleness	Spearman's rho	0,009002	0,05746
Link with hornet impact			
Correlation with colony decline during winter	Kendall's tau	**0,00939**	
Correlation with colony decline during winter	Spearman's rho	**0,009002**	
Association with winter survival	Kruskal-Wallis	0,5351	
Association with hornet predation observation	Kruskal-Wallis	0,3255	

No association was found between the response to the hornet and the intensity of sun exposure (Kruskal-Wallis test: $\chi^2 = 5{,}5638$; df = 2; $P = 0{,}06192$) or between the response and occupying a double-stacked hive (Kruskal-Wallis test: $\chi^2 = 0{,}96661$; df = 1; $P = 0{,}3255$) in the data of 2023.

Honeybee responses to the test objects

In the data of 2022, the hornet elicited a significantly stronger response (Mean: 1,519) than the piece of wood (Mean: 0,2308, Wilcoxon rank sum test: $W = 2468$; $P < 0,0001$) or the rod containing queen bee pheromone (Mean: 0,2692, Wilcoxon rank sum test: $W = 2433,5$; $P < 0,0001$). No significant correlation was found between the response to the hornet and the response to either the piece of wood (Kendall's tau: $\tau = -0,008272886$; $P = 0,9511$, Spearman's rho: $\rho = -0,008581844$; $P = 0,9519$) or the pheromone (Kendall's tau: $\tau = -0,1320694$; $P = 0,3237$, Spearman's rho: $\rho = -0,1382052$; $P = 0,3285$). Similarly, in the data of 2023, the response to the hornet (Mean: 1,591) was significantly higher than to the piece of wood (Mean: 0,4318, Wilcoxon rank sum test: $W = 1664,5$; $P < 0,0001$) or the pheromone (Mean: 0,1818, Wilcoxon rank sum test: $W = 1749,0$; $P < 0,0001$), and no significant correlation was observed between the response to the hornet and the response to the piece of wood (Kendall's tau: $\tau = 0,05383077$; $P = 0,7069$, Spearman's rho: $\rho = 0,05733283$; $P = 0,7116$) or the pheromone (Kendall's tau: $\tau = 0,05715896$; $P = 0,6869$, Spearman's rho: $\rho = 0,06037222$; $P = 0,6971$).

Figure 4: Boxplot comparing the distribution of response to the hornet scores across different gentleness levels in Apis mellifera mellifera colonies in the data of 2023.

In the data of 2023, a negative correlation between the response to the hornet and gentleness approached significance (Kendall's tau: $\tau = -0,2612747$; $P = 0,05184$, Spearman's rho: $\rho = -0,2885894$; $P = 0,05746$). Although the results did not reach statistical significance, there appears to be a visible trend (Figure 4). In contrast, this was less pronounced in the data of 2022 (Kendall's tau: $\tau = 0,208694$; $P = 0,09787$, Spearman's rho: $\rho = 0,225034$; $P = 0,1087$).

Influence of maternal lineage on honeybee responses

Analysis of the 2023 dataset revealed an association between the response to the hornet and maternal lineage (Kruskal-Wallis test: $\chi^2 = 21,118$; df = 10; $P = 0,02029$). In contrast, no such association was found in the data of 2022 (Kruskal-Wallis test: $\chi^2 = 2,5821$; df = 4; $P = 0,6300$). No association was found between gentleness and maternal lineage in the 2022 data (Kruskal-Wallis test: $\chi^2 = 2,8584$; df = 4; P = 0,5818) or in the data of 2023 (Kruskal-Wallis test: $\chi^2 = 11,366$; df = 10; $P = 0,3297$).

Link between honeybee response and hornet impact

Data from 2022 indicated that the response to the hornet was negatively correlated with colony decline during winter (Kendall's tau: τ = -0,3025132; P = 0,00939, Spearman's rho: ρ = -0,3587887; P = 0,009002). However, no significant correlation was found with winter survival (Kruskal-Wallis test: χ^2 = 0,38462; df = 1; P = 0,5351) or active predation by hornets being observed at the hive (Kruskal-Wallis test: χ^2 = 2,4469; df = 1; P = 0,1178).

Survey experiments

Results of the association tests conducted on the survey data are summarized in Table 2.

General information on the participants

A total of 87 respondents participated in the survey. The survey reached beekeepers from various regions in Belgium and the Netherlands, with approximately half of the respondents (43 out of 87) residing in Limburg, Belgium.

The majority of participants (62,1 %) indicated that they had been keeping bees for no less than 10 years, and 79,3 % had been beekeeping for no less than 20 years (Figure 5b). Most participants (94,3 %) indicated that they keep bees as a hobby, while only one participant (1,1 %) reported beekeeping as his or her main occupation (Figure 5c). With a majority of 64,4 %, *A. m. carnica* was the most kept honeybee variety among respondents (Figure 5d). 24,1 % indicated to work with the native *A. m. mellifera*. These percentages also include beekeepers working with more than 1 type of honeybee. Most participants (95,4 %) were aware that *A. m. mellifera* is the only native honeybee subspecies in Flanders. 97,7 % believed it is important to work with a honeybee variety that is very well adapted to local weather conditions.

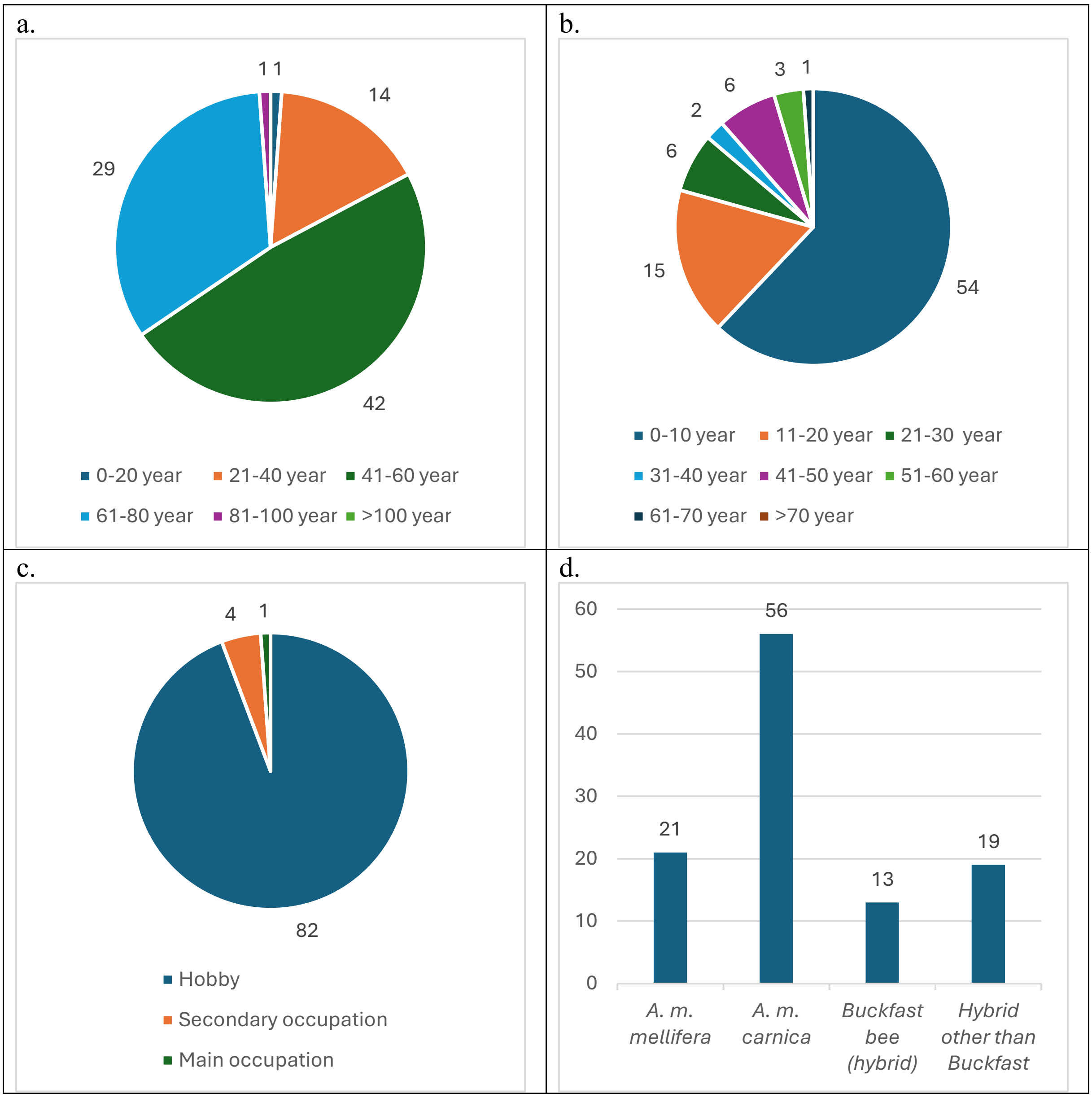

Figure 5: Visualisation of the proportions of age (a), years of beekeeping experience (b), the beekeepers' purpose—commercial, secondary occupation or hobby—of beekeeping activities (c) and honeybee varieties used (d) among participants of the survey. Note that some beekeepers work with multiple honeybee varieties, resulting in the total number of honeybees used exceeding the total number of respondents.

Valuation of defensibility and gentleness in the context of the invasive Asian hornet

51,7 % of respondents reported experiencing colony losses last winter. Among those, one-fifth (20,0 %) addressed *Vespa velutina nigrithorax* as being a cause for their losses. 47,1 % indicated being negatively affected by this invasive hornet species, which can be associated with their place of residency (Fisher's exact test: $P < 0,0001$).

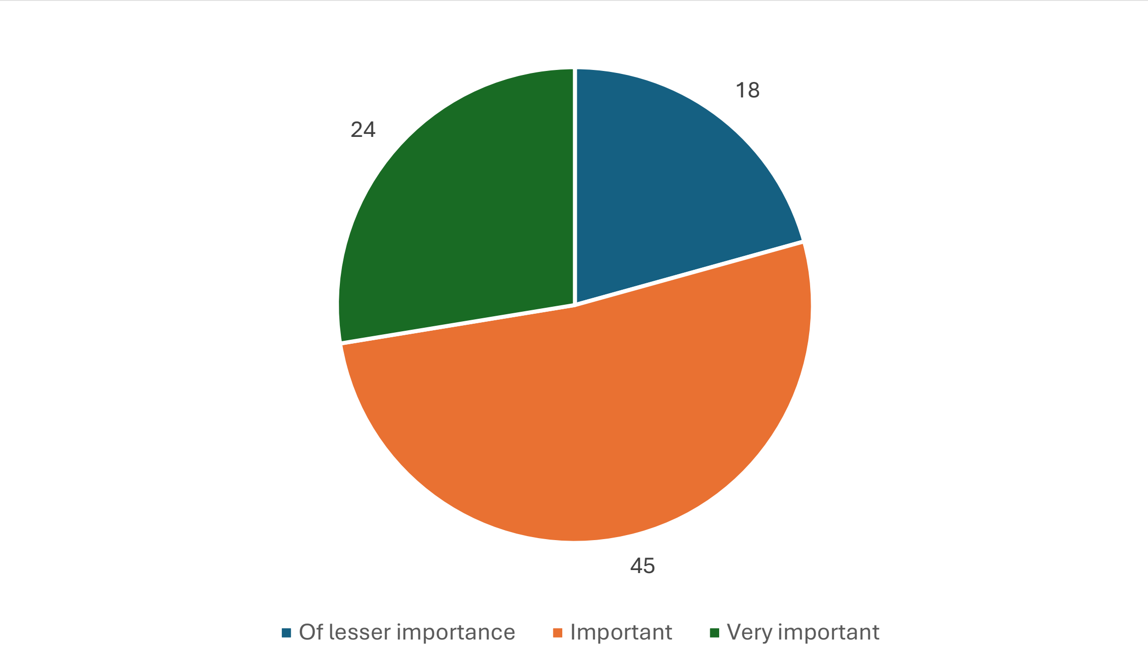

Figure 6: Proportions of importance attributed to honeybee gentleness towards the beekeeper, as rated by participants of the survey.

A total of 79,3 % of respondents valued gentleness in honeybees towards the beekeeper as an important trait, with 27,6 % considering it very important (Figure 6). Conversely, 20,7 % indicated that this trait was of lesser importance to them. A majority of 96,6 % of participants expressed a willingness to work with bee colonies that are better able to defend against *V. v. nigrithorax*. Only 40,2 % would remain willing to work with such colonies if they also exhibited aggression towards the beekeeper, which is significantly lower (McNemar test: $\chi^2 = 47,02$; df = 1; $P < 0,0001$). Beekeepers who are more willing to work with colonies that exhibit both strong defensive capabilities and aggression towards the beekeeper generally tended to place less importance on gentleness as a trait (logistic regression: SE = 0,3809; df = 1; $P = 0,000741$). An association between the value a participant attributed to colony gentleness and the honeybee varieties kept by the beekeeper approached significance (Fisher's exact test: $P = 0,06421$). Repeating the analysis using only data from beekeepers working exclusively with one honeybee variety yielded a significant result (Fisher's exact test: $P = 0,04194$).

27,6 % of participants indicated that they might cease beekeeping if bee colonies are unable to defend themselves against *V. v. nigrithorax* without beekeeper intervention. Within the group of participants that indicated to have lost colonies last winter, the number of beekeepers indicating they might cease beekeeping was significant higher among those who indicated *V. v. nigrithorax* as a cause of colony loss (Fisher's exact test: $P = 0,04275$).

Table 2: Association tests conducted on the survey data. Significant outcomes are highlighted in bold.

Variables tested between	Statistical test	*P*-value
Willingness to work with defensive but aggressive colonies and **attributed value to colony gentleness**	Logistic regression	**0,000741**
Importance of working with a locally adapted honeybee variety and **attributed value to colony gentleness**	Logistic regression	0,9970
Willingness to work with defensive but aggressive colonies and **age**	Logistic regression	0,5520
Willingness to work with defensive but aggressive colonies and **years of beekeeping experience**	Logistic regression	0,8400
Willingness to work with defensive but aggressive colonies and **purpose of beekeeping activities**	Logistic regression	0,9910
Ceasing beekeeping if colonies can't defend themselves against the Asian hornet and **age**	Logistic regression	0,7150
Ceasing beekeeping if colonies can't defend themselves against the Asian hornet and **years of beekeeping experience**	Logistic regression	0,6866
Ceasing beekeeping if colonies can't defend themselves against the Asian hornet and **purpose of beekeeping activities**	Logistic regression	0,6020
Willingness to work with defensive but aggressive colonies and **being negatively affected by the Asian hornet**	Chi-square test	0,6632
Ceasing beekeeping if colonies can't defend themselves against the Asian hornet and **being negatively affected by the Asian hornet**	Chi-square test	0,5675
Having experienced colony losses last winter and **being negatively affected by the Asian hornet**	Chi-square test	0,8997
Ceasing beekeeping if colonies can't defend themselves against the Asian hornet and **willingness to work with defensive but aggressive colonies**	Chi-square test	0,6794
Willingness to work with defensive but aggressive colonies and **having experienced colony losses last winter**	Chi-square test	0,5412
Willingness to work with defensive but aggressive colonies and **being negatively affected by the Asian hornet**	Fisher's exact test	1
Being negatively affected by the Asian hornet and **postal code**	Fisher's exact test	**< 0,0001**
Willingness to work with defensive but aggressive colonies and **honeybee varieties kept**	Fisher's exact test	0,3047

Willingness to work with defensive but aggressive colonies and **importance of locally adapted honeybee variety**	Fisher's exact test	0,5135
Ceasing beekeeping if colonies can't defend themselves against the Asian hornet and **honeybee varieties kept**	Fisher's exact test	0,2714
Attributed value to colony gentleness and **honeybee varieties kept**	Fisher's exact test	0,06421
Attributed value to colony gentleness and **honeybee varieties kept** (subset with one variety)	Fisher's exact test	**0,04194**
Willingness to work with defensive but aggressive colonies and **indicating the Asian hornet as cause of colony losses**	Fisher's exact test	0,2604
Ceasing beekeeping if colonies can't defend themselves against the Asian hornet and **indicating the Asian hornet as cause of colony losses**	Fisher's exact test	**0,04275**
Attributed value to colony gentleness and **age**	Spearman rank correlation test	0,5304
Attributed value to colony gentleness and **years of beekeeping experience**	Spearman rank correlation test	0,4080
Attributed value to colony gentleness and **purpose of beekeeping activities**	Spearman rank correlation test	0,2724

Discussion

Variation between colonies suggests a potential for selection

The dark European honeybee, *Apis mellifera mellifera*, has experienced a strong declines within its native range DE LA RUA et al. (2009); MEIXNER et al. (2013). Invasion of the invasive hornet *Vespa velutina nigrithorax* is an additional challenge for its conservation, as its presence reduces foraging activity and negatively impacts winter survival of the honeybee colonies MONCEAU et al. (2018). Dark European honeybee colonies differ in expression of defensive behaviour RUTTENER (1988); UZUNOV et al. (2014). By exposing them to a dead *V. v. nigrithorax*, this study aimed to assess the defensive behaviour of native dark European honeybee colonies in Limburg to the invasive Asian hornet. The results of this study reveal variation in the responses of colonies to the presented hornet, ranging from no reaction to strong interest involving multiple bees engaging with the hornet. In both the data collected in 2022 and 2023, no link between study site and the response of a colony on the hornet was found, indicating variation among colonies is not defined by the study site. Since data was collected per location, other factors such as the time of day, sunlight exposure and a possible increase or decrease of pheromones on objects during the experiment—the dead hornet may still have contained pheromones which might have decreased while preforming measurements, and bees could possibly leave pheromones on objects—are not expected to have had a strong impact on the variation observed, as these factors could have exerted influence on the link between the response towards the hornet and the study site. However, analysis of the dataset of 2023 revealed an association between this response and the maternal lineage, suggesting that the defensive behaviour against *V. v. nigrithorax* has a genetic component. This is consistent with an earlier study indicating that in *A. m. mellifera* defensive behaviour is heritable trait GUICHARD et al. (2020). The heritable variation in the defensive response to the hornet offers potential for both natural selection and human-mediated selection programs to enhance the defensive capabilities of the dark European honeybee against the invasive Asian hornet. Interestingly, no association with maternal lineage was observed in the data of 2022. This discrepancy suggests that the results should be interpreted with caution due to potential issues such as insufficient sample size or confounding factors.

Recognition of the invasive Asian hornet as threat

V. velutina is present in Limburg as an invasive species since 2018 SCHOONVAERE et al. (2020). At the time of the experiment in 2023, active predation by the Asian hornet had not yet been observed at the colonies in Bosland National Park, suggesting that these colonies may not have previously encountered the hornet. No associations were found between the response to the dead Asian hornet and the responses to either the piece of wood or the queen bee pheromone. Additionally, the hornet elicited a significantly stronger response than both control objects. This may indicate that the observed reaction may not be solely attributable to the presence of a silhouette or an odour, which cautiously suggests that the dark European honeybees are able to distinguish the hornet as a greater threat. During an experiment of KEN et al. (2005), the temperature obtained during balling was lower after 5 minutes for a false hornet, made of plastic and painted to mimic *V. velutina*, compared to a living hornet, but no difference in initial reaction after 15 seconds was proved. While the subspecies of *A. mellifera* used in this previous study was not specified, this may imply that the initial response to the dead hornet—considering it as a good replica—in this study could be similar to that of a living one. This, combined with a stronger response to the hornet compared to the piece of wood and no association being found between these objects, may suggest *A. m. mellifera* is able to visually recognise *V. v. nigrithorax*. This may be influenced by similarities the invasive predator has with its native predator *Vespa crabro* MONCEAU et al. (2013). Regarding pheromones, there is less certainty due to the unknown quantity and type of pheromones remained on the dead hornet used during the experiment. Further research is needed to clarify the role of pheromones in the defensive behaviour of *A. m. mellifera* against *V. v. nigrithorax*.

Stronger defensive responses may reduce predation pressure

Analysis of the dataset of 2022 revealed a negative association between colony decline during winter and the strength of response to the hornet. This suggests that a stronger defensive reaction can reduce the predation pressure exerted by the Asian hornet. In the presence of multiple beehives, *Vespa velutina* does not make prey choices randomly or based on the amount of food, such as colony size or stored resources, and is known to preferentially target honeybee species that are less capable of defending themselves when more than one species is present TAN et al. (2007); MONCEAU et al. (2014b). In combination with the results of this study this may indicate that also within a species or subspecies the Asian hornet preferentially targets beehives exhibiting lower levels of defensive behaviour compared to those with stronger defensive responses. However, no associations were found between the strength of response to the hornet and winter survival or whether active predation by the hornet has been observed. While predation by *V. v. nigrithorax* may negatively impact colonies, winter survival is influenced by multiple factors other than the Asian hornet NEUMANN & CARRECK (2010). In addition, even when a colony might be successful in killing *V. Velutina* foragers, the hornets could still pose a potential risk as they can possibly be a new vector to transmit pathogens between honeybee colonies YANG et al. (2020). The lack of an association with the observation of active predation might be due to the possibility that not all predation events were observed, or because the recorded observations do not fully capture the intensity of predation, which can vary between hives MONCEAU et al. (2014b).

During this study, no balling behaviour was observed. To date, balling behaviour in the dark European honeybee had only been observed when a hornet enters the hive or comes into contact with bees guarding the nest entrance MONCEAU et al. (2018).

Possible link between defensibility and gentleness

Intensive selection by beekeepers for gentleness in honeybees may negatively impact their defensive capabilities against predators UZUNOV et al. (2014). Conversely, gentleness is also an important trait to consider, as beekeepers may import other honeybee varieties if they deem the native honeybee less favourable. This has been a key factor in the decline of the dark European honeybee within the last century REINSCH et al. (1991); DE LA RUA et al. (2009); UZUNOV et al. (2014). This highlights a potential conflict between selecting for gentleness and the ability to defend against the Asian hornet. In this study, the response of dark European honeybee colonies to the presented hornet was compared with their level of gentleness to assess a potential association between defence against *V. v. nigrithorax* and gentleness towards beekeepers. The data of this study does not support a link between the strength of the response to the hornet and gentleness in *A. m. mellifera*. However, the data of 2023 shows a visible trend between these traits (Figure 4). Existence of a negative association between the response to the Asian hornet and gentleness towards the beekeeper can therefore not be excluded.

No association was found between gentleness and maternal lineage. This contrasts with previous research indicating that gentleness in *A. m. mellifera* has a genetic basis and, moreover, that it has also been subject to selection by beekeepers RUTTENER (1988); UZUNOV et al. (2014); GUICHARD et al. (2021). This may suggest that the results should be interpreted with caution due to potential issues such as insufficient sample size or confounding factors.

Survey

The survey reached beekeepers from various regions in Belgium and the Netherlands, including answers of beekeepers working with honeybee varieties other than the dark European honeybee. Sampling cannot be considered random, hence the results are not representative of the beekeeping population in Limburg or any other region. However, the results could still provide indicative information about beekeepers' attitudes towards honeybee gentleness and defensive behaviours.

While participants generally expressed willingness to work with honeybee colonies that could better defend themselves against *V. v. nigrithorax*, most indicated to be not willing to work with such colonies if they also exhibited aggression towards the beekeeper. These findings suggest that, even in the context of the invasive *V. v. nigrithorax*, gentleness is still considered a valuable trait by many beekeepers. Additionally, among beekeepers who work exclusively with a single honeybee variety, an association was found between the value placed on gentleness and the variety of honeybee worked with. This suggests that beekeepers' preference for gentleness may influence their choice of honeybee variety. The data shows no association between the commercial importance of beekeeping—whether as a primary profession, a secondary occupation, or a hobby—and the value attributed to gentleness. This observed lack of association may be less meaningful due to the insufficient data available from non-hobbyist beekeepers.

Among respondents who reported colony losses last winter, one-fifth identified *Vespa velutina nigrithorax* as a contributing factor. This reflects that, while the Asian hornet can act as an additional stressor, winter survival of honeybee colonies is influenced by multiple factors NEUMANN & CARRECK (2010). Reporting being negatively affected by the invasive hornet is found to be associated with the place of residence, which aligns with a heterogeneous regional distribution of *V. v. nigrithorax* BESSA et al. (2016). Another possible explanation could be that Limburg was not yet fully colonized by the invasive hornet species. The survey indicated that if honeybee colonies appeared unable to defend themselves against the Asian hornet without beekeeper intervention, this can be a reason for beekeepers to consider discontinuing beekeeping. This suggests that a reduction in the number of beekeepers might be anticipated. Data of this survey suggests this tendency may be more pronounced among those who attributed their colony losses to the invasive hornet.

Recommendations for further research

This study did not find a clear link between defensive behaviour against the Asian hornet and gentleness towards the beekeeper in the dark European honeybee. Results of the survey reflect that, even in the context of the invasive Asian hornet, gentleness may remain a valued trait for many beekeepers. This underscores the need to understand the potential conflict between selecting for gentleness and effective defensive capabilities against *V. v. nigrithorax*, which is crucial for developing an effective breeding strategy.

Hybridization with other subspecies leads to increased defensive behaviour in *A. m. mellifera* RUTTENER (1988); UZUNOV et al. (2014); OSTROVERKHOVA et al. (2024). This behaviour is often described in the context of defensive reactions against beekeepers, but it may also extend to defence against hornets. This raises an intriguing question for further research: does a link between the defensive response of *A. m. mellifera* to *V. v. nigrithorax* and the degree of hybridization exist? As the maximum mating range of the dark European honeybee extends its conservation aera in Bosland National Park and beekeepers working with honeybees other than the dark European honeybee are present in the region, a limited chance of mating with other subspecies still exits JENSEN et al. (2005); ELEN (2017); BOSLAB (2021-2023). If the results of this study could be combined with genetic data, it could elucidate whether a link between the response to the Asian hornet and the degree of hybridization might exist. Existence of such an association could have an impact on conservation of the dark European honeybee, because the presence of this invasive hornet might influence selection in favour of hybrids.

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