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ORIGINAL RESEARCH ARTICLE



# Swarming, defensive and hygienic behaviour in honey bee colonies of different genetic origin in a pan-European experiment

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## Summary

Honey bee colonies exhibit a wide range of variation in their behaviour, depending on their genetic origin and environmental factors. The COLOSS Genotype-Environment Interactions Experiment gave us the opportunity to investigate the phenotypic expression of the swarming, defensive and hygienic behaviour of 16 genotypes from five different honey bee subspecies in various environmental conditions. In 2010 and 2011, a total of 621 colonies were monitored and tested according to a standard protocol for estimation of expression of these three behavioural traits. The factors: year, genotype, location, origin (local vs. non-local) and season (only for hygienic behaviour) were considered in statistical analyses to estimate their effect on expression of these behaviours. The general outcome of our study is that genotype and location have a significant effect on the analysed traits. For all characters, the variability among locations was higher than the variability among genotypes. We also detected significant variability between the genotypes from different subspecies, generally confirming their known characteristics, although great variability within subspecies was noticed. Defensive and swarming behaviour were each positively correlated across the two years, confirming genetic control of these characters. Defensive behaviour was lower in colonies of local origin, and was negatively correlated with hygienic behaviour. Hygienic behaviour was strongly influenced by the season in which the test was performed. The results from our study demonstrate that there is great behavioural variation among different subspecies and strains. Sustainable protection of local genotypes can be promoted by combining conservation efforts with selection and breeding to improve the appreciation by beekeepers of native stock.

# Comportamiento higiénico, defensivo y enjambrador en colonias de la abeja de la miel con diferente origen genético en un experimento pan-europeo

## Resumen

Las colonias de abejas de la miel exhiben una amplia gama de variaciones en su comportamiento, en función de su origen genético y de factores ambientales. El estudio paneuropeo realizado en el marco de la acción COST "COLOSS" nos dio la oportunidad de investigar la expresión fenotípica de la enjambrazón, de los comportamientos defensivo e higiénico de 16 genotipos de 5 subespecies diferentes de abejas bajo distintas condiciones ambientales. En 2010 y 2011 un total de 621 colonias iniciales fueron controladas y examinadas de acuerdo con un protocolo estándar para la estimación de la expresión de estos tres rasgos de comportamiento. Los factores año, genotipo, ubicación, origen (local frente a no-local) y estación (sólo para el comportamiento higiénico) fueron considerados en los análisis estadísticos para estimar su efecto sobre la expresión de estos comportamientos. El resultado general de nuestro estudio es que el genotipo y la ubicación tienen un efecto significativo sobre las variables analizadas. Para todos los caracteres, la variabilidad entre las localidades fue mayor que la variabilidad entre los genotipos. Sin embargo, también se detectó una variabilidad significativa entre los genotipos de las diferentes subespecies, lo que en general confirma sus características conocidas. Los resultados de nuestro estudio demuestran la necesidad de apoyar los esfuerzos locales de cría para la conservación sostenible de las poblaciones europeas de la abeja de la miel.

**Keywords:** COLOSS, Genotype-Environment Interactions Experiment, *Apis mellifera* L., honey bee, behaviour, swarming, defensive, hygienic

## Introduction

The honey bee colony is a complex society which commands a wide range of behaviours to protect itself from predators and diseases and to enable its reproduction and survival (Winston, 1987). Some of these behaviours are of significance for the interests of beekeepers and thus have been recognised in selection and breeding programmes (Ruttner, 1972).

One of the most important behaviours for the honey bee colony is reproduction. In honey bees, there are two levels of reproduction: the individual level (mating and oviposition) and the colony level (division or multiplication of the colony, generally known as swarming). Another well-known type of behaviour in honey bees is colony defence consisting of recognition of predators, alerting nestmates and enacting anti-predator behaviour (Collins *et al.*, 1980; Moritz *et al.*, 1987; Breed *et al.*, 2004). Beekeepers have for a long time recognised these two behaviours, swarming and colony defence (Crane, 1990), and enacted breeding strategies to reduce their expression, in opposition to natural selection (Ruttner, 1972; Möbus, 1983; Villumstad, 1983; Poklukar, 1999; Moritz and Southwick, 1992). For example, the natural way for honey bee colonies to reproduce is to swarm, and this behaviour is thus intimately connected to fitness, but in contrast to this, beekeepers favour colonies that never swarm. Likewise, defensive behaviour is not favoured by beekeepers, but very docile honey bee colonies can easily fall prey to natural enemies, like wasps, birds or mammals. Hence, maintaining honey bees with optimal behaviour from a beekeeping point of view, at the same time maintains the demand for continuous artificial selection, at least until fixation occurs, i.e. unfavourable traits

are removed entirely from a population. Such fixation, however, has not been achieved, which is a strong argument for the idea that honey bees should not be considered as "domesticated".

More recently, behaviours related to colony health and disease control, such as hygienic behaviour and grooming have gained more interest among selection programmes (Rothenbuhler, 1964; Gilliam *et al.*, 1983, 1988; Spivak, 1996; Boecking and Spivak, 1999; Büchler *et al.*, 2010; Rinderer *et al.*, 2010). This type of artificial selection seems to support natural selection, as increasing hygienic or grooming behaviour should help the bees to remove several pathogens and parasites otherwise causing diseases. However, the fact that bees show variability for the expression of such traits can suggest that either no strong fitness values are attached to them or that an optimum colony composition exists, which is based on the distribution of workers to various tasks (Robinson, 1992; Tofts and Franks, 1992; Page and Mitchell, 1998).

Environmental factors and beekeeping managing techniques can strongly influence the expression of these behavioural traits (Winston, 1987; Delaplane *et al.*, 2013; Büchler *et al.*, 2013). Nonetheless, they are known to vary characteristically among the numerous honey bee subspecies and populations that have been scientifically described so far (Adam, 1968; Ruttner, 1988a; Sheppard *et al.*, 1997; Sheppard and Meixner, 2003). Although the description and discrimination of honey bee subspecies and populations is based on morphometric and molecular data (reviewed in Meixner *et al.*, 2013), specific and characteristic behavioural patterns are known from many honey bee populations (Adam, 1968; Ruttner 1988a, 1992). While some subspecies exhibit patterns considered favourable from the point of view of bee-

keepers, and in consequence have been highly selected and widely distributed, other subspecies with behaviours regarded as unfavourable are being endangered or may have already been replaced by introduced populations. The selection, high queen production and now almost worldwide distribution of *A. m. ligustica* and *A. m. carnica*, due to their comparatively weak defensive behaviour, combined with ready spring buildup and high honey production (de la Rúa *et al.*, 2005), is a good example. In several regions of Europe, these two subspecies are now favoured over the native honey bees such as *A. m. mellifera* and *A. m. siciliana* which were dismissed by beekeepers for several reasons, such as their more pronounced defensive behaviour and high tendency to swarm, respectively. However, recently, conservation and reintroduction efforts have been initiated for some of these populations (Jensen *et al.*, 2005; Dall'Olio *et al.*, 2007; Strange *et al.*, 2008; de la Rúa *et al.*, 2009). The starting point for all conservation efforts is the recognition and a precise description of the endangered population or subspecies (Bouga *et al.*, 2011). In addition to a discrimination based on morphometric and/or molecular data, this also needs to include an assessment of behavioural traits.

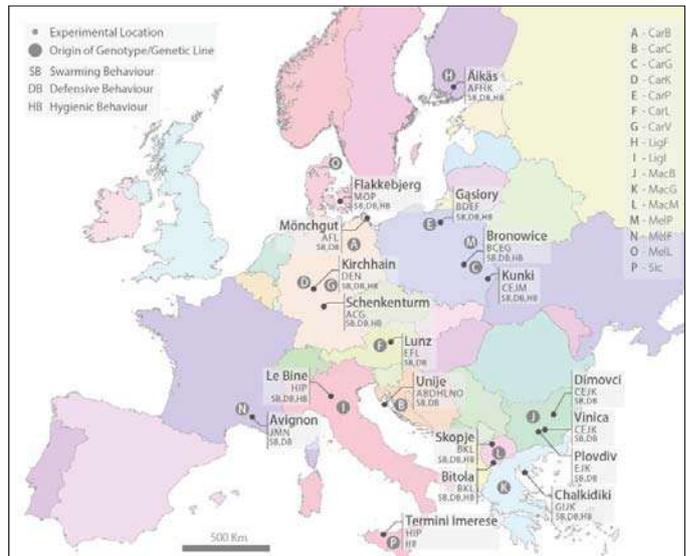
It is not known to what extent genotype, environment and local adaptation influence the expression of behavioural traits. With the COLOSS Genotype-Environment Interactions Experiment (Costa *et al.*, 2012) we had a unique chance to study the effects of genotype and environment on the expression of defensive, swarming and hygienic behaviours on a large scale.

## Material and method

### Experiment design

The experiment started in the late summer 2009 and ran until 31 March 2012. A starting total of 621 colonies was established in 21 different test apiaries across Europe, and 16 genotypes (detailed description in Francis *et al.*, 2014) from several European subspecies of honey bees (Car - *A. m. carnica*, Lig - *A. m. ligustica*, Mac - *A. m. macedonica*, Mel - *A. m. mellifera*, Sic - *A. m. siciliana*) were included (Fig. 1). In two of the 21 apiaries, however, no behavioural data were collected at all. The apiary of Toulouse (France) was given up soon after the start of the experiment. At the apiary of Probistip (Macedonia) all colonies died in the first winter, and therefore, no behavioural assessments could be taken. In the remaining 19 locations, it was not possible to completely measure all of the traits in each apiary, so the data presented in this paper originate from 18 apiaries for swarming and defence behaviour, and from 12 apiaries for hygienic behaviour. Details on location and genotype distribution, and the tests performed, are given in Fig. 1.

The test apiaries were distributed across various European climates and biomes, spanning from the Mediterranean to Finland. Meteorological data for each location were obtained for 2010, and enormous variation of climatic conditions was observed (for details see Hatjina *et al.*, 2014).



**Fig. 1.** Map of Europe showing the 19 test locations. The name of each location is given in the white box, together with the genotypes and the behavioural traits assessed. The legends at left and right top link the abbreviations to the genotypes and the tests that were performed. Adapted from Francis *et al.*, 2014.

For instance, the number of days with minimum temperatures below 0°C ranged from zero in Termini Imerese (Sicily) to 174 in Äikäs (Finland). Similar magnitudes existed for other meteorological parameters, which as a whole affect the opportunities to forage and the availability of food.

For each genotype, the declared subspecies, location of origin and degree of breeding efforts are summarised in Table 1, together with the abbreviation used in this paper. Each test apiary consisted of colonies belonging to at least three different genotypes, the one of local origin and two or more non-local ones. Queens that were superseded in the course of the experiment were considered as belonging to the original genotype. Several training sessions were organised and a common protocol was developed to standardise colony management, evaluation procedures and timing of colony measurements (censuses). Further details of the experiment design and the colony evaluation protocol are given by Costa *et al.* (2012).

### Methods for testing behavioural parameters

The evaluation of swarming, defensive and hygienic behaviour was carried out during the active seasons of 2010 and 2011. The behavioural traits were evaluated every time the colonies in the apiary were visited and also at each colony census (three censuses per year). The swarming tendency was assessed by assigning a score to each colony according to the standard four point system of Ruttner (1972), where the score of 4 indicated that no swarming tendency was noticeable, and 1 indicated that the colony swarmed or that swarming could be prevented only by extensive intervention. Defensive behaviour was also evaluated using a score system, as this is the method commonly used in European breeding programmes and as it was shown to be the most, reliable

**Table 1.** Genotype abbreviation, declared subspecies, locations of origin and degree of breeding efforts. <sup>1</sup>Bantin, <sup>2</sup>Sisak, <sup>3</sup>Pulawy, <sup>4</sup>Kirchhain, <sup>5</sup>Gasiory, <sup>6</sup>Lunz, <sup>7</sup>Veitshöchheim, <sup>8</sup>Paimio, <sup>9</sup>Emilia Romagna, <sup>10</sup>Plovdiv, <sup>11</sup>Chalkidiki, <sup>12</sup>Probistip, <sup>13</sup>Augustowska, <sup>14</sup>Avignon, <sup>15</sup>Læsø, <sup>16</sup>Eolie Islands, Sicily, n.a. - no available information.

Abbr.	Subspecies	Location of origin	Strong breeding efforts	Abbr.	Subspecies	Location of origin	Strong breeding efforts
CarB	<i>A. m. carnica</i>	Germany <sup>1</sup>	Yes	LigI	<i>A. m. ligustica</i>	Italy <sup>9</sup>	Yes
CarC	<i>A. m. carnica</i>	Croatia <sup>2</sup>	Yes	MacB	<i>A. m. macedonica</i>	Bulgaria <sup>10</sup>	Yes
CarG	<i>A. m. carnica</i>	Poland <sup>3</sup>	Yes	MacG	<i>A. m. macedonica</i>	Greece <sup>11</sup>	No
CarK	<i>A. m. carnica</i>	Germany <sup>4</sup>	Yes	MacM	<i>A. m. macedonica</i>	Macedonia <sup>12</sup>	No
CarP	<i>A. m. carnica</i>	Poland <sup>5</sup>	Yes	MelP	<i>A. m. mellifera</i>	Poland <sup>13</sup>	Yes
CarL	<i>A. m. carnica</i>	Austria <sup>6</sup>	Yes	MelF	<i>A. m. mellifera</i>	France <sup>14</sup>	n.a.
CarV	<i>A. m. carnica</i>	Germany <sup>7</sup>	Yes	MelL	<i>A. m. mellifera</i>	Denmark <sup>15</sup>	n.a.
LigF	<i>A. m. ligustica</i>	Finland <sup>8</sup>	Yes	Sic	<i>A. m. siciliana</i>	Italy <sup>16</sup>	Yes

method when compared to other field assays (Guzmán-Novoa *et al.*, 2003). Likewise, in the score system utilised for, 4 indicated no need of protection or smoke to avoid stings, and 1 indicated that maximum protection was necessary (Ruttner, 1972). Specific definitions for assigning gradual scores for both behavioural traits have been laid down in the common protocol (Costa *et al.*, 2012).

For evaluation of the colonies' hygienic behaviour we used the "pin-test" (Costa *et al.*, 2012; Büchler *et al.*, 2010, 2013), where 50 cells containing white- or pink-eyed pupae are pierced through the cell capping with an entomological pin size n° 2 (diameter = 0.45 mm). The removal of the killed pupae by the adult bees was estimated after a time interval of 8 to 10 hours. The pin-test was repeatedly performed through the entire active season. The honey production data from Hatjina *et al.* (2014) was used to determine the existence of association between the studied behavioural traits and honey yield.

## Data processing and statistical analysis

Results of swarming and defence behaviour tests were referred to as one mean value of each colony per year. In case of hygienic behaviour, repeated measures were collected. All records of swarming, defensive and hygienic behaviour were subject to pre-statistical description and dispersion evaluation. Data for hygienic behaviour performed close to normal distribution. In swarming tendency and defensive behaviour we assumed normality since pre-tested transformations did not significantly improve normality. The final evaluation was performed by Generalized Linear Model (GLM) analysis, where different models were used.

In evaluation of swarming and defensive behaviour of each colony the model includes year, location, genotype and origin (local vs. non-local origin) as fixed factors, while in the case of hygienic behaviour besides those factors the fixed effect of seasons was added, defined as spring (which included tests performed in March and April), May, June, July and August and autumn (tests performed in September and October). As for evaluation of survival (Büchler *et al.*, 2014), development (Hatjina *et al.*, 2014) and pests and diseases (Meixner *et al.*, 2014) the

interaction of the genotype with the environment was represented by the factor "origin", in which performance of colonies in their area of origin was compared to colonies out of their of origin. The adjusted means of all fixed factors with a significant influence were compared with a Bonferroni test using the Mean Square Error of the estimation. To assess the association of the traits with each other and with honey production, Pearson correlation coefficients were calculated on the base of the observed means for each colony per year. All statistical analyses were done in SPSS, v20.0 software.

## Results

The results of the GLM analyses of swarming, defensive and hygienic behaviour for the biologically most relevant factors are given in Table 2.

### Swarming behaviour

The GLM analysis showed that swarming behaviour was highly significantly affected ( $p < 0.01$ ) by year, location and genotype. In contrast, the origin of the genotype (local vs. non-local) was not found to have a significant effect on the trait (Table. 2). The expression of swarming behaviour was significantly ( $p < 0.05$ ) higher in the second year of the experiment when the original queens were two years old. In the first and second year, the adjusted mean score values for swarming were  $3.23 \pm 0.07$  and  $2.94 \pm 0.11$ .

The variability among locations was much higher than among genotypes. The adjusted mean values of swarming behaviour scores in the different locations ranged from  $1.08 \pm 0.19$  in Kirchhain to  $3.79 \pm 0.23$  in Lunz, whereas variation among genotypes ranged from  $2.62 \pm 0.17$  for MacG to  $3.55 \pm 0.49$  for Sic (Tables 3 and 4). Accordingly, we observed a considerably lower variation of the swarming trait - when compared to the locations - between the genotypes within the same subspecies (for those that were represented by several genotypes): within *A. m. macedonica* MacB differed significantly from MacG ( $p < 0.05$ ), and within *A. m. mellifera* MelP differed significantly from MelF ( $p < 0.05$ ).

**Table 2.** Results of GLM analyses of swarming, defensive and hygienic behaviour.

Swarming behaviour				Defensive behaviour				Hygienic behaviour			
Source	df	Mean Square	F	Source	df	Mean Square	F	Source	df	Mean Square	F
<b>Model</b>	35	163.91	173.94**	<b>Model</b>	35	152.10	417.09**	<b>Model</b>	34	32756.33	64.68**
<b>Year</b>	1	7.59	8.05**	<b>Year</b>	1	0.00	0.00	<b>Year</b>	1	685.97	1.35
<b>Location</b>	17	13.81	14.66**	<b>Location</b>	17	5.94	16.28**	<b>Season</b>	5	2624.59	5.18**
<b>Genotype</b>	15	2.18	2.32**	<b>Genotype</b>	15	2.20	6.03**	<b>Location</b>	11	4872.37	9.62**
<b>Origin</b>	1	0.64	0.67	<b>Origin</b>	1	5.41	14.84**	<b>Genotype</b>	15	1116.99	2.21**
<b>Error</b>	527	0.94		<b>Error</b>	535	0.37		<b>Origin</b>	1	761.68	1.50
<b>Total</b>	562			<b>Total</b>	570			<b>Error</b>	778	506.48	
								<b>Total</b>	812		
<b>R<sup>2</sup> = 0.92 (Adjusted R<sup>2</sup> = 0.92)</b>				<b>R<sup>2</sup> = 0.97 (Adjusted R<sup>2</sup> = 0.96)</b>				<b>R<sup>2</sup> = 0.74 (Adjusted R<sup>2</sup> = 0.73)</b>			

**Table 3.** Colonies' adjusted means values (LS mean), standard errors, number and percentage of locations with significant difference of swarming behaviour score (1-4) by location.

Location	LS Mean±SE	No. (%) of locations with significant difference
Lunz	3.79 ± 0.23	6 (35.3)
Dimovci	3.54 ± 0.27	2 (11.8)
Plovdiv	3.00 ± 0.32	1 (5.9)
Vinica	3.72 ± 0.33	2 (11.8)
Unije	2.82 ± 0.17	4 (23.5)
Āikās	3.47 ± 0.18	2 (11.8)
Bitola	3.18 ± 0.33	1 (5.9)
Skopje	3.38 ± 0.33	1 (5.9)
Kirchhain	1.08 ± 0.19	17 (100)
Mönchgut	2.15 ± 0.20	11 (64.7)
Schenkenturm	2.80 ± 0.23	3 (17.6)
Chalkidiki	3.63 ± 0.19	2 (11.8)
Le Bine	2.55 ± 0.30	5 (29.4)
Bronowice	3.02 ± 0.17	5 (29.4)
Kunki	3.71 ± 0.19	6 (35.3)
Gasiory	3.54 ± 0.16	3 (17.6)
Flakkebjerg	2.59 ± 0.42	1 (5.9)
Avignon	3.61 ± 0.27	4 (23.5)

Overall, the genotypes MacG, MelL and CarC were most prone to swarm, while the expression of the trait in Sic, LigF and CarK was very low.

### Defensive behaviour

Location, genotype and origin had a highly significant effect (GLM,  $p < 0.01$ ) on defensive behaviour. The trait was expressed consistently in both years, so that year was not a significant factor ( $p > 0.05$ ) (Table 2). As with the results obtained for swarming, the level of variability among the locations was higher than among the genotypes. The adjusted mean values of defensive behaviour scores in locations ranged from  $1.94 \pm 0.12$  in Chalkidiki to  $3.91 \pm 0.16$  in Skopje, and across the genotypes they ranged from  $2.45 \pm 0.21$  for MelP to  $3.71 \pm 0.31$  for Sic (Tables 5 and 6).

**Table 4.** Colonies' adjusted means values (LS mean), standard errors, number and percentage of genotypes with significant difference and multiple comparison of the genotypes for swarming behaviour score (1-4). \*The significant differences among genetic origins are reported with "+" indicating positive ranking and "-" negative ranking towards compared genotypes. For reasons of space the reciprocals are not reported.

Genotype	LS Mean±SE	No. (%) of genotypes with significant difference	Post-Hoc analysis The mean difference is significant at the 0.05 level*
CarB	3.13 ± 0.21	1 (6.6)	-MacB
CarC	2.72 ± 0.18	1 (6.6)	-MacB
CarG	3.15 ± 0.21	1 (6.6)	+MelF
CarK	3.45 ± 0.20	1 (6.6)	-MacB
CarP	3.18 ± 0.13	2 (13.3)	-MacB, +MelF
CarL	2.87 ± 0.16	0 (0)	
CarV	2.75 ± 0.20	1 (6.6)	-MacB
LigF	3.50 ± 0.22	1 (6.6)	+MelF
LigI	2.92 ± 0.29	1 (6.6)	-MacB
MacB	3.30 ± 0.17	9 (60)	+MacG, +MelF, +MelL
MacG	2.62 ± 0.17	2 (13.3)	+MelF
MacM	3.27 ± 0.21	1 (6.6)	+MelF
MelP	3.25 ± 0.34	1 (6.6)	+MelF
MelF	3.07 ± 0.24	7 (46.6)	
MelL	2.67 ± 0.41	1 (6.6)	
Sic	3.55 ± 0.49	0 (0)	

A high level of variability was noticed within the subspecies: for example, a marked variability within *A. m. carnica* (represented by 7 genotypes) was confirmed by post-hoc analysis, with significant differences between CarG and CarP, and CarK and CarP. Significant differences were also detected within *A. m. ligustica* (LigF vs. LigI) and *A. m. mellifera* (MelL towards MelP and MelF), but not within *A. m. macedonica*.

The lowest scores (corresponding to the highest expression of defensive behaviour) were found in MelP and MelF. In contrast, the

**Table 5.** Colonies' adjusted means values (LS mean), standard errors, number and percentage of locations with significant difference of defensive behaviour score (1-4) by location.

Location	LS Mean±SE	No. (%) of locations with significant difference
Lunz	3.20 ± 0.15	2 (11.8)
Dimovci	2.91 ± 0.17	3 (17.6)
Plovdiv	3.26 ± 0.22	2 (11.8)
Vinica	3.07 ± 0.21	1 (5.9)
Unije	3.78 ± 0.11	7 (41.2)
Äikäs	2.86 ± 0.11	6 (35.3)
Bitola	3.70 ± 0.18	6 (35.3)
Skopje	3.91 ± 0.16	7 (41.2)
Kirchhain	2.59 ± 0.12	7 (41.2)
Mönchgut	3.36 ± 0.12	4 (23.5)
Schenkenturm	2.73 ± 0.14	6 (35.3)
Chalkidiki	1.94 ± 0.12	16 (94.1)
Le Bine	2.19 ± 0.19	10 (58.8)
Bronowice	3.48 ± 0.11	6 (35.3)
Kunki	2.79 ± 0.12	6 (35.3)
Gasiory	3.34 ± 0.10	5 (29.4)
Flakkebjerg	3.35 ± 0.26	2 (11.8)
Avignon	3.22 ± 0.17	2 (11.8)

**Table 6.** Colonies' adjusted means values (LS mean), standard errors, number and percentage of genotypes with significant difference and multiple comparison of the genotypes for defensive behaviour score (1-4). \*The significant differences among genetic origins are reported with "+" indicating positive ranking and "-"negative ranking towards compared genotypes. For reasons of space the reciprocals are not reported.

Genotype	LS Mean±SE	No. (%) of genotypes with significant difference	Post-Hoc analysis The mean difference is significant at the 0.05 level*
CarB	3.14 ± 0.13	2 (13.3)	+LigI, +MelF
CarC	2.78 ± 0.11	3 (20)	+LigI, +MelP, +MelF
CarG	3.41 ± 0.13	5 (33.3)	+CarP, +LigI, +MacG, +MelP, +MelF
CarK	3.41 ± 0.12	5 (33.3)	+CarP, +LigI, +MacG, +MelP, +MelF
CarP	2.83 ± 0.08	5 (33.3)	-CarL, +LigI, -MelL
CarL	3.11 ± 0.10	5 (33.3)	+LigI, +MacG, +MelP, +MelF
CarV	3.33 ± 0.13	2 (13.3)	+LigI, +MelF
LigF	3.07 ± 0.14	1 (6.6)	+LigI
LigI	3.15 ± 0.18	13 (86.6)	-MacB, -MacG, -MacM, -MelL, -Sic
MacB	3.36 ± 0.11	2 (13.3)	-MelL
MacG	3.08 ± 0.11	5 (33.3)	-MelL
MacM	2.68 ± 0.13	2 (13.3)	+MelF
MelP	2.45 ± 0.21	5 (33.3)	-MelL, -Sic
MelF	2.62 ± 0.15	8 (53.3)	-MelL, -Sic
MelL	3.35 ± 0.25	6 (40)	
Sic	3.71 ± 0.31	3 (20)	

**Table 7.** Colonies' adjusted means values (LS mean), standard errors and multiple comparison of the seasons for hygienic behaviour score (1-4). \*The significant differences among seasons are reported with "+" indicating positive ranking and "-"negative ranking towards compared season. For reasons of space the reciprocals are not reported.

Season	LS Mean±SE	Post-Hoc analysis The mean difference is significant at 0.05 level*
Spring	38.36 ± 4.80	+May, +July
May	30.34 ± 3.01	-August, -Autumn
June	34.74 ± 1.88	-August
July	40.04 ± 2.56	-August
August	49.73 ± 3.42	
Autumn	32.93 ± 3.39	

highest mean values, corresponding to the most docile genotypes were found to be Sic, CarK and CarG.

Origin of the genotype (local vs. non-local) was found to have a highly significant effect ( $p < 0.01$ ) on the colonies' expression of defensive behaviour (Table 2): genotypes assessed at their location of origin expressed a lower defensiveness (higher score)  $3.20 \pm 0.06$  compared to non-local genotypes with a score of  $2.98 \pm 0.05$ .

## Hygienic behaviour

The GLM analysis showed that hygienic behaviour was not significantly affected by year or origin. The trait was instead highly significantly ( $p < 0.01$ ) affected by season, location and genotype (Table 2). The highest expression of the trait was estimated in July and August, while the lowest was estimated in May and autumn (Table 7).

As for the previous traits, the variability among the locations was much higher than among the genotypes. The adjusted mean values of removal rates across the locations ranged from  $19.53 \pm 8.59$  in Bitola to  $76.14 \pm 7.86$  in Flakkebjerg. Among the genotypes the range was  $15.32 \pm 9.80$  in MelL to  $49.15 \pm 3.57$  in CarV (Tables 8 and 9).

Some intra-subspecies significant variability ( $p < 0.05$ ) for this behavioural trait was detected in *A. m. carnica* (CarP vs. CarV), *A. m. ligustica* (LigI vs. LigF) and *A. m. mellifera* (MelL vs. MelF). Within *A. m. macedonica* we observed no significant variability. Overall, the lowest expression of the trait was estimated for MelL, MelF and MacM and the highest for the genotypes CarV, MacB and CarL.

## Correlations

The relationships between the score of each trait in the two years, between the scores of all traits in the two years, and of all traits with honey production within each of the test years, were estimated and are given in Table 10. Across the two test years (2010 and 2011), we found significant ( $p < 0.01$ ) positive moderate correlations for the swarming behaviour ( $r = 0.36$ ) and for the defensive behaviour ( $r = 0.47$ ). In contrast, we found that the scores for hygienic behaviour of the two test years were not significantly correlated. In both years, the

defensive behaviour was significantly negatively correlated with hygienic behaviour. In 2010 the correlation between the traits was weak ( $r = -0.17$ ;  $p < 0.05$ ), but in the second year the association was moderate with a correlation coefficient of  $-0.34$  ( $p < 0.01$ ) (Table 10). In 2010, the removal rate was estimated to be significantly positively weakly correlated ( $p < 0.05$ ) with honey production.

**Table 8.** Colonies' adjusted means values (LS mean), standard errors, number and percentage of locations with significant difference of hygienic behaviour (removal %) by location.

Location	LS Mean $\pm$ SE	No. (%) of locations with significant difference
Äikäs	19.71 $\pm$ 3.85	4 (36.4)
Bitola	19.53 $\pm$ 8.59	4 (36.4)
Skopje	31.68 $\pm$ 9.47	2 (18.2)
Kirchhain	37.69 $\pm$ 4.48	4 (36.4)
Schenkenturm	25.46 $\pm$ 3.99	4 (36.4)
Chalkidiki	41.57 $\pm$ 3.28	9 (81.8)
Le Bine	69.34 $\pm$ 5.55	9 (81.8)
Termini	56.00 $\pm$ 6.47	7 (63.6)
Bronowice	22.76 $\pm$ 3.41	4 (36.4)
Kunki	21.61 $\pm$ 3.48	4 (36.4)
Gasiory	30.78 $\pm$ 3.63	4 (36.4)
Flakkebjerg	76.14 $\pm$ 7.86	9 (81.8)

**Table 9.** Colonies' adjusted means values (LS mean), standard errors, number and percentage of genotypes with significant difference and multiple comparison of the genotypes for hygienic behaviour (removal %). The significant differences among genetic origins are reported with "+" indicating positive ranking and "-" negative ranking towards compared genotypes. For reasons of space the reciprocals are not reported.

Genotype	LS Mean $\pm$ SE	No. (%) of genotypes with significant difference	Post-Hoc analysis The mean difference is significant at the 0.05 level*
CarB	42.28 $\pm$ 4.65	4 (26.6)	-LigI, -MacB, -Mell, -Sic
CarC	40.99 $\pm$ 3.91	3 (20)	-LigI, -Mell, -Sic
CarG	41.56 $\pm$ 3.69	4 (26.6)	-LigI, -MacB, -Mell, -Sic
CarK	38.74 $\pm$ 4.45	2 (13.3)	-LigI, -Sic
CarP	34.77 $\pm$ 3.29	5 (33.3)	-CarV, -LigI, -MacB, -Mell, -Sic
CarL	42.48 $\pm$ 4.16	2 (13.3)	-LigI, -Sic
CarV	49.15 $\pm$ 3.57	3 (20)	-LigI, -Sic
LigF	41.30 $\pm$ 4.86	2 (13.3)	-LigI
LigI	41.98 $\pm$ 4.27	12 (80)	+MacB, +MacG, +MacM, +Melf
MacB	47.56 $\pm$ 4.14	6 (40)	+Melf, -Sic
MacG	35.31 $\pm$ 3.70	2 (13.3)	-Sic
MacM	30.38 $\pm$ 10.97	2 (13.3)	-Sic
MelP	40.79 $\pm$ 7.40	0 (0)	
MelF	24.49 $\pm$ 7.10	4 (26.6)	-Mell, -Sic
MelL	15.32 $\pm$ 9.80	5 (33.3)	
Sic	35.96 $\pm$ 6.42	12 (80)	

**Table 10.** Correlation index between swarming, defensive and hygienic behaviour traits and honey production for each observation year.

\*\*Correlation is significant at the 0.01 level (2-tailed). \*Correlation is significant at the 0.05 level (2-tailed).

		Swarming behaviour 2011	Defensive behaviour 2010	Defensive behaviour 2011	Hygienic behaviour 2010	Hygienic behaviour 2011	Honey Production 2010	Honey Production 2011
Swarming behaviour 2010	Pearson Correlation	0.36**	0.01	0.03	0.03	0.05	-0.09	-0.11
	Sig. (2-tailed)	0.000	0.823	0.741	0.732	0.616	0.085	0.192
	N	146	410	158	191	97	359	155
Swarming behaviour 2011	Pearson Correlation		0.02	0.16*	-0.06	-0.03	-0.20*	0.11
	Sig. (2-tailed)		0.796	0.049	0.575	0.796	0.022	0.216
	N		145	147	80	78	136	135
Defensive behaviour 2010	Pearson Correlation			0.47**	-0.17*	-0.09	0.04	-0.18*
	Sig. (2-tailed)			0.000	0.022	0.360	0.464	0.030
	N			157	192	96	358	154
Defensive behaviour 2011	Pearson Correlation				-0.24*	-0.34**	-0.25**	-0.13
	Sig. (2-tailed)				0.023	0.002	0.003	0.139
	N				91	79	148	138
Hygienic behaviour 2010	Pearson Correlation					-0.08	0.17*	0.07
	Sig. (2-tailed)					0.488	0.023	0.567
	N					86	193	74
Hygienic behaviour 2011	Pearson Correlation						-0.33**	-0.22
	Sig. (2-tailed)						0.002	0.059
	N						85	73
Honey Production 2010	Pearson Correlation							0.32**
	Sig. (2-tailed)							0.000
	N							148

## Discussion

Our results show that the colonies we compared showed high variability in the expression of the swarming, defensive and hygienic behaviour traits. The factor exerting the strongest influence was location, which can be seen as the sum of all abiotic and biotic components in a given environment. The length of the active season, which in our study varied from four up to 10 months, together with food availability, significantly affected development trajectories of the colonies at different locations (Hatjina *et al.*, 2014), and this may have affected not only colony development but also performance.

As detailed in Costa *et al.* (2012), the colonies at all locations were managed according to a common protocol that specified certain compulsory procedures, and timing and methods of assessments and sampling. Beyond these key activities, however, the colonies were managed according to the locally prevailing beekeeping practice, which therefore also contributed to the influence exerted by the factor location. The influence of the factor genotype was found to be generally weaker in comparison to location, yet in many cases it was significant.

The colonies in our experiment showed a stronger inclination to swarm in the second year. This is not surprising, as most of the colonies still had their original queens, and it is well known that colonies with older queens have a higher swarming tendency (Winston, 1987; Free, 1987; Uzunov, 2013). Although the variability of this trait was greater among locations than genotypes, the genetic influence is demonstrated by the positive correlation between the annual scores of the colonies ( $r = 0.36$ ;  $p < 0.01$ ). It has indeed been shown by several studies that different honey bee populations may express different levels of expression of this trait (Adam, 1968; Ruttner, 1988a, 1992).

Among the tested strains, the colonies from the MacB genotype, corresponding to *A. m. macedonica* from Bulgaria, showed a significantly lower swarming behaviour compared to the majority of the remaining genotypes. These findings are in consistency with observations of Brother Adam (1968) and Ruttner (1988a) who reported that a low swarming tendency is one of the main values of the Macedonian honey bees. However, within the *A. m. macedonica* from our study, we found considerable variation: low swarming tendency in the Bulgarian population, higher in the Greek one, and intermediate in the Macedonian population. This probably reflects the wide range of the *A. m. macedonica* origin which was covered, and could also be due to the fact that the MacB genotype originated from a long-term breeding programme which considered swarming tendency as a selective trait (Petrov, 2010). However, it must be noted that most of the genotypes (62.5%) used in our experiment originated from selected stock, but considerable variation in the swarming trait was retained.

A significantly higher expression of swarming tendency was detected in the colonies of the MelF genotype, originating from a population in France that was reported as "varroa surviving bees", although the conclusion was that swarming did probably not play a major role in

varroa resistance (Le Conte *et al.*, 2007). A high swarming tendency of *A. m. mellifera* was reported by Brother Adam (1968) in both Northern and Southern populations (Cooper, 1986; Ruttner, 1988a), and the differences we observed between MelF and genotypes belonging to the evolutionary lineage C (CarG, CarP, LigF, MacB, MacG and MacM), confirm these reports. The lack of long-lasting intensive artificial selection in this somewhat neglected subspecies has probably contributed to retaining the natural expression of this trait.

Interestingly, the genotype representing *A. m. siciliana* in our experiment did not show the high swarming tendency which we expected according to observations by other authors: Ruttner (1988a) reports *A. m. siciliana* as being characterised by a very high swarming tendency, as also confirmed by Tiemann and Brückner (1993). Evidently, the conservation programme enacted on the Eolian islands, which has saved this subspecies from extinction (Dall'Olio *et al.*, 2008), may have selected for reduction of expression of this trait.

We observed a strong genetic influence on the defensive behaviour of the colonies, which is confirmed by a significant correlation between the annual scores of the colonies ( $r = 0.47$ ;  $p < 0.01$ ). In accordance with the wide range of geographic origins represented, a high level of variability for this trait was noticed within the subspecies: for example, significant differences were observed between CarG and CarP, and also between CarK and CarP. These behavioural differences within *A. m. carnica* may to some extent reflect the heterogeneity observed at the genetic level, as reported by Francis *et al.* (2014).

The most docile genotypes in our experiment were found to be Sic, CarK and CarG. *A. m. carnica* is generally known and appreciated for its docile temperament (Ruttner, 1986, 1988a, 1992; de la Rúa *et al.*, 2005; Gregorc and Lokar, 2010). Furthermore, the genotypes CarK and CarG originate from long term breeding programmes in Germany and Poland, respectively. The policy on selection and breeding among the beekeeping associations of these countries place high value on low defensive behaviour which therefore has been maintained as one of the main selection goals.

In the case of *A. m. siciliana*, our findings are in agreement with the reports by Ruttner (1988a) that the Sicilian bee is "rather gentle and quiet when manipulated", but contrast with many reports from Sicilian beekeepers, who complain that "the local black bee is aggressive". However, it must be noted that these reports most likely refer to colonies that are hybridised with *A. m. ligustica*, which has been massively imported into the island since the 1980s, while almost pure *A. m. siciliana* has survived as a small population on conservation islands (Longo, 1984; Dall'Olio *et al.*, 2008). A growing group of beekeepers now using bees from the conservation programme confirm the low aggressiveness of the pure bred black bees, but also report an increasing defensiveness in following generations, if pure mating is not achieved (Sapienza, pers. comm.). Thus, our study supports the hypothesis that one consequence of hybridisation is an increase of defensive behaviour. This phenomenon was indeed observed in successive generations of

other crosses, e.g. *A. m. carnica* x *A. m. mellifera* as described by Ruttner (1988b) and the three-way hybrid (*A. m. ligustica* x *A. m. caucasica*) x *A. m. mellifera* described by Fresnaye and Lavie (1976). Our observations show that beekeeping with the native bee need not necessarily be hampered by defensive bees, as long as hybridisation with different genetic origins is avoided.

In many countries, *A. m. mellifera* suffers from a negative reputation among beekeepers for its bad temper. Our findings appear to support this estimation in that colonies of the genotypes MelP and MelF were the most defensive ones in the experiment. However, both these genotypes were also found to be highly hybridised (Francis *et al.*, 2014), which may have aggravated their tendency to defensiveness. While the MelF population has for decades not been subjected to artificial selection (Le Conte *et al.*, 2007), the MelP genotype from Poland is being maintained by open mating only, which appears insufficient for keeping foreign alleles from introgressing (Francis *et al.*, 2014).

In contrast, the third *A. m. mellifera* genotype in the experiment, Mell originating from a small conservation area on the Danish island of Læsø (Jensen *et al.*, 2005), showed a significantly less pronounced defensive behaviour compared to MelP and MelF. This genotype was also found to be comparatively pure, showing considerably less hybridisation with other subspecies (Francis *et al.*, 2014; Pinto *et al.*, 2014).

The lack of coordinated selection efforts within *A. m. mellifera* is clearly manifest in the significantly lower scores for defensive behaviour, which, on the other hand, may provide a straightforward explanation for its unpopularity among beekeepers in large portions of its native area. Nonetheless, as discussed for the case of *A. m. siciliana* above, it appears that also in *A. m. mellifera* defensiveness is strongly related to hybridisation, where purer populations are also considerably more docile (Ruttner, 1988b; Fresnaye and Lavie, 1976). The hybridisation effect on defensive behaviour could result from insufficient queen mandibular pheromone levels circulating among the workers (Gervan *et al.*, 2005), maybe as a result of either variance of queen's release of pheromone or workers' expectations (Naumann *et al.*, 1991; Pankiv *et al.*, 1994).

Our results also show a significant interaction between genotype and location, since colonies of local origin were considerably less defensive than introduced ones. This could indicate that the non-local genotypes expressed stronger defensive reactions due to the lack of adaptation with environmental conditions, such as higher or lower temperatures than in their local range, or the presence of different predators (Arechavaleta-Velasco and Hunt, 2003; Breed *et al.*, 2004). The lower defensiveness of local bees could also be a consequence of the management adaptation to local bees, where, for instance, the testers may have more experience in managing the local bees and use techniques disturbing the bees to a lesser extent. (Southwick and Moritz, 1987; Breed *et al.*, 2004).

When considering the whole test period, the genotypes originating

from the Apennine Peninsula and Sicily (LigI and Sic) were the ones with the most differences in hygienic behaviour compared to the other genotypes. However, the genotype which displayed the highest cleaning rate was CarV, closely followed by MacB. These results are not surprising, as these genotypes originate from breeding programmes with intensive selection for hygienic behaviour (Petrov, 2010). However, other genotypes, also originating from breeding programmes which include hygienic behaviour as a selective trait (e.g. the Polish genotypes CarP, CarG and MelP), exhibited hygienic scores that were lower than expected from previous reports (Panasiuk *et al.*, 2008; Bąk *et al.*, 2010), highlighting the strong influence of environmental factors (location, season) on this trait.

Seasonal differences in expression of hygienic behaviour have been frequently reported (Panasiuk *et al.*, 2009; Güler and Toy, 2013), but there are also contradictory reports (Bigio *et al.*, 2013). It is likely that season and location interact to yield unique combinations of floral availability and nectar flow, which are known to influence the expression of hygienic behaviour (Momot and Rothenbuhler, 1971; Robinson, 1992; Spivak and Gilliam, 1998a,b; Johnson, 2003). Furthermore, according to several authors, hygienic behaviour is mainly exhibited by workers that are younger than three weeks (Thompson, 1964; Arathi *et al.*, 2000; Panasiuk *et al.*, 2010), and different balance of young and old bees in the colony may affect expression the trait at the colony level. This may, for example, explain the lower cleaning rate we observed in autumn.

In the first observation year, we found a significant, but weak correlation between hygienic behaviour and honey production. This is in agreement with reports that during a good nectar flow bees remove dead brood faster, thereby preparing cells for nectar collection (Thompson, 1964; Momot and Rothenbuhler, 1971; Spivak *et al.*, 1995; Spivak and Reuter, 1998). In contrast, in adverse weather conditions, the activity of foragers drops, leading to lower pollen and honey stores and subsequently affecting brood rearing and the structure of the bee colony (Mattila and Otis, 2006). In the research of Garcia *et al.* (2013) hygienic behaviour showed a high correlation with honey production. A nectar inflow on the day before hygienic behaviour is measured may also enhance cleaning rates (Panasiuk *et al.*, 2009).

We estimated a significant negative correlation between the scores of defensive and hygienic behaviour in both test years, which may suggest that defensive bees are more hygienic. This would be in line with observations reported by beekeepers and also some published research that defensive bees tend to be more hygienic (Winston, 1995; Paleolog, 2009). However, Rinderer (1986) and Kefuss *et al.* (1996) did not find correlations between hygienic and defensive behaviours in different strains of European honey bees.

In conclusion, our results demonstrate that the behaviours of swarming, colony defence and brood hygiene are significantly affected by both, environmental and genetic factors. For defensive behaviour we also observed a significant interaction between genotype and environment, in that local genotypes were significantly more docile

than non-local ones. We interpret this as an expression of adaptation to stressors present in the local environment. Not surprisingly, the highest values for the observed characters were expressed by genotypes originating from breeding or conservation programmes, thus showing success of selection for the behaviours that beekeepers desire.

In contrast, the range of these behaviours expressed by the *A. m. mellifera* genotypes clearly illustrate a lack of coordinated selection and breeding efforts (Ruttner, 1990; Gallmann, 2012). Thus, they provide an explanation for the low appreciation of this subspecies among beekeepers, which lead to a state of endangerment and near extinction in large areas of its native range (Winston, 1987, de la Rúa *et al.*, 2005).

From a practical point of view, the strong environmental and genetic effects reveal the need for intensive exploration of the available behavioural variation among different subspecies and strains. Our results also show that a sustainable protection of local genotypes can most likely be promoted and improved if conservation efforts are combined with selection and breeding from native stock to improve its appreciation by beekeepers of the respective regions.

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