Der Einfluss von Wirtsfaktoren der Honigbiene (*Apis mellifera* L.) auf den Reproduktionserfolg der parasitischen Milbe *Varroa destructor* (Anderson & Trueman) und die Auswirkungen einer horizontalen Verbreitung des Parasiten auf den Befall der Bienenvölker.

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1 Einleitung

Die parasitische Bienenmilbe *Varroa destructor* wird weltweit als Hauptursache für periodisch auftretende Völkerverluste der westlichen Honigbiene *Apis mellifera* angesehen (Boecking and Genersch, 2008; Brodschneider et al., 2010; Chauzat et al., 2010; Guzmán-Novoa et al., 2010). Schon ein relativ geringer Varroabefall der Bienen von ca. 6% im Spätherbst reicht aus, um die Überwinterung der Bienenvölker zu gefährden (Genersch et al., 2010). Die Varroose stellt nicht nur ein wirtschaftliches Problem für die Imkerei dar, sondern ist auch eine ökonomische und ökologische Bedrohung für die Kulturlandschaft, da die Bestäubung vieler Nutz- und Wildpflanzen direkt von der Honigbiene abhängt (Watanabe, 1994; Klein et al., 2007; Rucker et al., 2012).

Das ursprüngliche Verbreitungsgebiet der Varroamilbe befindet sich in Südostasien, wo sie in Völkern der östlichen Honigbiene *Apis cerana* vorkommt, hier jedoch keine bedrohlichen Schäden verursacht. Ein Wirtswechsel von der asiatischen auf die westliche Honigbiene (Oldroyd, 1999) und weiträumige Transporte der infizierten Bienenvölker trugen zu einer globalen Verbreitung des Parasiten innerhalb einer kurzen Zeitspanne bei und hatten zur Folge, dass *V. destructor* in den Siebzigerjahren des letzten Jahrhunderts auch in Europa und Deutschland eingeführt wurde (Rosenkranz et al., 2010). Dies hatte und hat dramatische Folgen für wildlebende und bewirtschaftete *A. mellifera* Bienenvölker. Durch die rasche Verbreitung der Milbe und eine weitgehend fehlende natürliche Selektion konnte sich in Europa bisher kein stabiles Wirt-Parasit-System einstellen. Zudem fehlen nach wie vor Forschungsergebnisse der Bienenwissenschaft und praktische Erfahrungen in der Imkerschaft, um diesen neuen Parasiten nachhaltig zu kontrollieren. Die Bekämpfung von *V. destructor* wird nach wie vor als größte Herausforderung der modernen Imkerei angesehen (Dietemann et al., 2012), da weltweit nahezu kein varroafreies Volk mehr zu finden ist und zumindest in den gemäßigten Breiten ein Bienenvolk ohne regelmäßige Varroabehandlung innerhalb kurzer Zeit eingeht (Korpela et al., 1992). Eine wirtschaftliche Imkerei ist somit ohne

Varroabekämpfung nicht mehr möglich. Daraus ergeben sich für die Imker nun aber ähnliche Probleme wie in der übrigen modernen Landwirtschaft: Hohe Kosten durch die Behandlung, Resistenzentwicklung beim Parasiten und das Risiko der Belastung von Bienenprodukten mit Akarizid-Rückständen (Wallner, 1999; Wallner and Fries, 2003). Die Suche nach neuen Möglichkeiten zur Lösung des Varroaproblems ist daher ein Schwerpunkt der aktuellen Honigbienenforschung.

Ein Ansatzpunkt ist die Suche nach einer varroatoleranten Honigbiene, jedoch hatten alle Versuche einer diesbezüglichen Selektion und Zucht bisher keinen durchschlagenden Erfolg (Büchler et al., 2010). Die natürliche Selektion spielt in den gemäßigten Breiten bei der Etablierung eines stabilen Wirt-Parasit-Systems kaum eine Rolle, da es so gut wie keine wildlebenden Honigbienenpopulationen gibt. Für die Honigbienenvölker in Imkerhand fehlen klare und durchführbare Selektionskriterien. Es gibt jedoch einige wenige Beispiele die zeigen, dass unter den Bedingungen einer natürlichen Selektion Populationen von *A. mellifera* ohne Varroabekämpfung überleben können (Büchler et al., 2010).

So hat sich nach über zehn Jahren natürlicher Selektion der Reproduktionserfolg der Varroamilben in kleinen isolierten Bienenpopulationen auf der Insel Gotland (Schweden) und in Südfrankreich im Vergleich zu nicht selektierten Bienenherkünften signifikant reduziert (Locke et al., 2012). Diese Toleranz gegenüber der Varroamilbe ist ein Merkmal des Honigbienenwirtes und keine Eigenschaft der lokalen Milbenpopulation (Fries and Bommarco, 2007).

Entscheidend für ein ausgeglichenes Wirt-Parasit-Verhältnis ist letztendlich die enorme Zunahme der Parasitenpopulation während der Saison zu unterbinden. Dabei spielen sowohl die Reproduktion der Varroaweibchen innerhalb der Brutzellen als auch Wechselwirkungen auf der Ebene des Bienenvolkes eine Rolle.

Der Lebenszyklus der weiblichen Milben ist in eine phoretische Phase auf den adulten Bienen und eine reproduktive Phase in der verdeckelten Brutzelle unterteilt. Kurz vor der Zellverdeckelung befällt die phoretische Milbe die Bienenbrutzellen und beginnt wenige Stunden später ihren Reproduktionszyklus. Dabei legt sie im Allgemeinen ein

männliches und mehrere weibliche Eier. Innerhalb der Brutzelle kommt es dann zu Bruder-Schwester-Paarungen der adulten Nachkommen (Rosenkranz et al., 2010). Allerdings ist nicht bei allen Brutmilben die Reproduktion erfolgreich: Neben zu später oder unvollständiger Eiablage gibt es auch Milbenweibchen, die zwar in Brutzellen eindringen, aber überhaupt keine Eier legen. Eine solche Infertilität der Varroaweibchen scheint der Hauptgrund für das stabile Wirt-Parasit-System beim ursprünglichen Wirt *A. cerana* zu sein: In Völkern des asiatischen Ursprungswirtes kann sich *V. destructor* nur in der Drohnenbrut vermehren (Boot et al., 1999; Rosenkranz et al., 2010).

Im Gegensatz dazu kann sich bei unseren Honigbienen (*A. mellifera*) die Varroamilbe sowohl in der Drohnen- als auch der Arbeiterinnenbrut fortpflanzen. Dadurch steigt die Milbenpopulation exponentiell an und der Milbenbefall kann sich infolge dessen im Frühjahr und Frühsommer innerhalb eines Monats verdoppeln (Fries et al., 1994). Doch auch bei *A. mellifera* sind 5-20 % der in die Brut eingedrungenen Milbenweibchen nicht in der Lage, Eier zu legen. Es konnte gezeigt werden, dass es sich hier um eine temporäre Infertilität der Varroaweibchen handelt, die wahrscheinlich durch Wirtsfaktoren verursacht wird. Bei der Aktivierung und Steuerung der Varroareproduktion spielen u.a. Substanzen der Wirtslarvenkutikula eine wichtige Rolle (Garrido and Rosenkranz, 2004). Allerdings ist die chemische Natur dieser Stoffe bisher noch nicht im Detail aufgeklärt. Die Kenntnis solcher Substanzen im Bienenvolk könnte entscheidend zur Entwicklung biologischer Bekämpfungskonzepte, die auf der Beeinflussung der Reproduktionssteuerung und Populationsentwicklung der Varroamilbe basieren, beitragen. Solche biologischen Bekämpfungsansätze könnten in Zukunft eine nachhaltige Alternative zu den momentan angewandten Behandlungsmöglichkeiten darstellen.

Die Populationsdynamik der Milbe hängt aber nicht nur vom Reproduktionserfolg der Varroaweibchen innerhalb der einzelnen Brutzellen ab; auch die Verbreitung der Varroamilben zwischen den Bienenvölkern könnte das Wachstum der Parasitenpopulation beeinflussen. Es gibt zwei grundsätzlich unterschiedliche Übertragungswege eines Parasiten, die häufig eine entscheidende Rolle bei der Etablierung eines stabilen Wirt-Parasit-Gleichgewichtes spielen (Fries and Camazine,

2001). Der vertikale Übertragungsweg (die Übertragung auf die Nachkommen) der Varroamilben im Bienenvolk ist die Übertragung von Mutter- zu Tochtervolk, wenn sich das Volk über das Schwärmen vermehrt. Unter natürlichen Bedingungen, ohne eine Massenaufstellung von Bienenvölkern, dürfte sich die Varroamilbe vor allem vertikal, also über den Schwarm, verbreiten. Dieser Übertragungsweg soll die Entwicklung hin zu einem stabilen Wirt-Parasit-Verhältnis favorisieren, um den Wirt in seiner Entwicklung nicht so einzuschränken, dass die weitere Verbreitung des Parasiten unterbunden wird. Eine horizontale Übertragung tritt zum einen innerhalb des Bienenvolkes auf, wenn die Varroamilben von Biene zu Bienen wechseln. Wichtiger ist jedoch die horizontale Verbreitung zwischen verschiedenen Völkern, wenn der Parasit durch Räuberei oder Verflug der Bienen von Volk zu Volk getragen wird.

In der heutigen Imkerei kommt es an attraktiven Standorten oft zu einer hohen Anzahl von Bienenvölkern, wodurch die horizontale Ausbreitung der Varroamilbe erleichtert wird (Greatti et al., 1992). Bisher fehlen allerdings belastbare Daten, um die Bedeutung dieser horizontalen Verbreitung auf die Populationsdynamik unter realistischen Feldbedingungen zu quantifizieren.

1.1 Ziel der Arbeit

In dieser Arbeit sollen auf unterschiedlichen Ebenen Faktoren analysiert werden, die einen Einfluss auf das Wachstum der Varroapopulation haben. Zunächst soll auf der individuellen Ebene untersucht werden, wie die Reproduktion der Varroaweibchen innerhalb der Brutzelle durch Faktoren der Wirtslarve beeinflusst wird. In einem neu etablierten Biotest wurde in Ein- und Umsetzversuchen in und zwischen Bienenbrutzellen *in vivo* untersucht, welche stadienspezifischen kutikulären Substanzen der Wirtslarve die Reproduktion der Varroamilbe beeinflussen. Insbesondere sollten die Ursachen für die „temporäre Infertilität“ der Varroaweibchen näher untersucht werden.

In einem weiteren Ansatz zum Thema „Varroainfertilität“ sollte in Kooperation mit dem Partnerinstitut in Halle an der Saale geprüft werden, ob tatsächlich - wie vermutet - genetisch determinierte Wirtsfaktoren für diese temporäre Infertilität verantwortlich

sind. Hierfür wurden in einem molekulargenetischen Ansatz mit Segregationsanalysen bei den haploiden Drohnen nach Genregionen gesucht, die mit der Infertilität der Varroaweibchen korreliert sind. Für die Analysen wurden Drohnen aus Hybridvölkern der vorselektierten „Gotland-Population“ (siehe oben) und der unselektierten Hohenheimer *A. mellifera carnica* Population verwendet. Das langfristige Ziel ist, über eine Identifizierung und Charakterisierung von Resistenzgenen in der Honigbiene ein DNA-basiertes Zuchtwerkzeug zu entwickeln, das es ermöglicht, präzise und mit geringem Aufwand Bienenvölker mit solchen Varroaresistenzgenen zu selektieren.

Im zweiten Teil dieser Arbeit sollten die Effekte einer horizontalen Verbreitung der Varroamilben auf die Populationsdynamik quantifiziert werden. Unter kontrollierten sowie unter praxisnahen Bedingungen wurden dabei Umfang und zeitlicher Verlauf der horizontalen Übertragung von Varroamilben („Varroainvasion“) in Abhängigkeit von Entfernung und Anzahl befallener Bienenvölker sowie das Vermehrungspotential der Milbe im Spätsommer und Herbst untersucht.

2 Veröffentlichungen

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Activation and interruption of the reproduction of *Varroa destructor* is triggered by host signals (*Apis mellifera*)

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ABSTRACT

The reproductive cycle of the parasitic mite *Varroa destructor* is closely linked to the development of the honey bee host larvae. Using a within colony approach we introduced phoretic *Varroa* females into brood cells of different age in order to analyze the capacity of certain stages of the honey bee larva to either activate or interrupt the reproduction of *Varroa* females. Only larvae within 18 h (worker) and 36 h (drones), respectively, after cell capping were able to stimulate the mite's oogenesis. Therewith we could specify for the first time the short time window where honey bee larvae provide the signals for the activation of the *Varroa* reproduction. Stage specific volatiles of the larval cuticle are at least part of these activation signals. This is confirmed by the successful stimulation of presumably non-reproducing mites to oviposition by the application of a larval extract into the sealed brood cells. According to preliminary quantitative GC–MS analysis we suggest certain fatty acid ethyl esters as candidate compounds.

If *Varroa* females that have just started with egg formation are transferred to brood cells containing host larvae of an elder stage two-thirds of these mites stopped their oogenesis. This confirms the presence of an additional signal in the host larvae allowing the reproducing mites to adjust their own reproductive cycle to the ontogenetic development of the host. From an adaptive point of view that sort of a stop signal enables the female mite to save resources for a next reproductive cycle if the own egg development is not sufficiently synchronized with the development of the host.

The results presented here offer the opportunity to analyze exactly those host stages that have the capacity to activate or interrupt the *Varroa* reproduction in order to identify the crucial host signals.

1. Introduction

The reproduction of the honey bee mite *Varroa destructor* is a crucial point for the population dynamic of this parasite (Fries et al., 1994; Calis et al., 1999). The life cycle of the female mites is subdivided into a phoretic phase on adult bees and a reproductive phase within worker or drone brood cells. For reproduction, the female mite leaves the adult bee and enters a brood cell with 5th instar larva shortly before the cell sealing and become stuck in the larval food at the bottom of the brood cell. Within a few hours after cell capping the larvae consume the rest of the food and set the mite free (reviewed in Rosenkranz et al. (2010)). At that time the female mite has already started with oogenesis in the terminal oocyte (Steiner et al., 1994; Garrido et al., 2000). In laboratory bioassays we have demonstrated that the first step of the activation of the mite's oogenesis is triggered by volatiles of the larval cuticle independently from the uptake of hemolymph by the mite (Garrido and Rosenkranz, 2004). The activating components are apparently in the polar fraction of the cuticular volatiles (Trouiller and Milani, 1999; Garrido and Rosenkranz, 2004). Additionally, stage specific factors of the host larvae and pupae, respectively, influence the sex of the mite's eggs (Garrido and Rosenkranz, 2003). Obviously, the activation as well as the course of the reproduction of *V. destructor* is closely correlated with the preimaginal development of the honey bee host. This may be an adaptation to the relatively short capping period of the honey bee worker brood. The female mite lays the first male egg approximately 70 h after cell capping followed by 3–5 female eggs in 30 h intervals (Martin, 1994; Rehm and Ritter, 1989). As the success of a reproductive cycle depends on the number of viable adult mated daughter mites that leave the brood cell together with the hatching young bee, the duration of the postcapping period is a limiting factor and, therefore, the mother mite should start egg laying as soon as possible (Rosenkranz et al., 2010).

However, the dependence of the mite reproduction from specific factors of the honey bee larvae offers possibilities for a host

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adaptation in order to limit the reproductive success of the parasite. So it is a long known phenomenon that a reasonable percentage of female mites do not reproduce successfully after invading a brood cell. Some mites do not lay eggs at all (reviewed in Rosenkranz et al. (2010), Carneiro et al. (2007), Correa-Marques et al. (2003) and Garrido and Rosenkranz (2003)), others do lay male or female eggs only or show delayed egg laying (Donzé et al., 1996; Martin et al., 1997; Locke and Fries, 2011). It is yet unknown to what degree host factors are responsible for those disorders in mite reproduction.

In our approach we focused on the phenomenon "non-reproduction" in *V. destructor*. At first, we proved in detail which larval instars are capable to induce the reproduction in *Varroa* females. Using a new within-colony bioassay we tried to verify if larval volatiles can activate the reproduction in those *V. destructor* females which actually are considered infertile. In a further approach we examined whether an already started oogenesis of *Varroa* females can be interrupted by the signals of certain host stages. Finally, we analyzed the pattern of cuticular compounds of those larval stages that have an influence on the fertility of the mite.

These experiments were performed to specify the role of stage specific signals of the host larvae for initiation and disruption of *Varroa* reproduction.

2. Materials and methods

2.1. Reproduction of V. destructor after natural infestation of brood cells

We used *A. mellifera carnica* colonies from the apiary at the University of Hohenheim headed by queens of our local breeding line. From June to September brood cells of drone and worker brood combs were individually analyzed for infestation with *V. destructor*. Only single infested brood cells containing 8–9 day old pupae with dark eyes and yellow thorax (Martin, 1994) were used for further analysis. Within those brood cells the female mites have already terminated egg laying but the first daughter mite has not finished the adult molt and can therefore easily be distinguished from the mother mite. All mites that have laid at least one egg were considered "reproductive", the mites without egg laying were considered "non-reproductive".

2.2. Artificial infestation of V. destructor into brood cells of different age

To determine the exact relation between larval age and activation of the *Varroa* reproduction, worker and drone brood cells were marked shortly before sealing on transparency sheets (Aumeier and Rosenkranz, 2001). Two to six hours later, capped brood cells were marked and considered "freshly capped". These marked brood cells were used for artificial infestation with phoretic *V. destructor* mites (Garrido and Rosenkranz, 2004). Mite-free colonies were chosen as host colony for these experiments to prevent the transfer of mites to brood cells that are already infested.

Phoretic mites for the infestation of the brood cells were sampled randomly from hive bees of heavily infested colonies but without clinical symptoms of Varroosis (Rosenkranz et al., 2010). Within 1 h the mites were introduced into the chosen brood cells. For that purpose, the cell capping was carefully folded out with a razor blade and the mite was introduced with an insect pin. Then the cell capping was closed again. Phoretic mites were introduced into freshly sealed brood cells and also into brood cells 6, 12, 18, 24 and 30 h after cell capping (worker brood) and 12, 24, 36, 48 and 60 h after cell capping (drone brood), respectively. The position of the treated brood cells on the combs was marked on transparency sheets. After artificial infestation all test combs were returned to the colony in order to guarantee the required temperature and humidity inside the brood cells. The treated combs remained in the colony for the entire duration of the experiment, because *V. destructor* mites introduced into brood cells and then kept in an incubator have lower reproduction rate compared to mites kept in the colony (Ibrahim and Spivak, 2006).

Eight days later the artificially infested brood cells were analyzed for reproduction of the introduced *V. destructor* female. Again, a *Varroa* mite was considered "reproductive" when at least one offspring (e.g. egg and/or nymphal stages) were present within the brood cell. Dead mites (in total <5% of the introduced mites) were not considered in the analysis.

2.3. Reproduction of Varroa mites that have been transferred into brood cells of different ages after initiation of oogenesis

Phoretic *V. destructor* mites were introduced into freshly capped worker brood cells (see Section 2.2 for details). 24 h after introduction into brood cells, one portion of these mites were transferred to cells of the same larval age (control, Fig. 1I) or to brood cells containing larvae that were 24 h (i.e. 48 h after cell capping, Fig. 1II) and 48 h (i.e. 72 h after cell capping, Fig. 1IV), respectively, older. Another portion of mites were transferred 48 h after introduction into freshly sealed brood cells to brood cells containing larvae that were 24 h older (i.e. 72 h after cell capping, Fig. 1III).

Only female mites which had already started with oogenesis were transferred. The successful start of the oogenesis can easily and clearly be defined by the swollen idiosoma of the introduced mother mite caused by the growth of the first oocyte (Martin, 1994; Garrido and Rosenkranz, 2003; Steiner et al., 1995). The infested cells were examined 8 days after cell capping. All reproductive stages, i.e. eggs, protonymphs, deutonymphs and males were recorded.

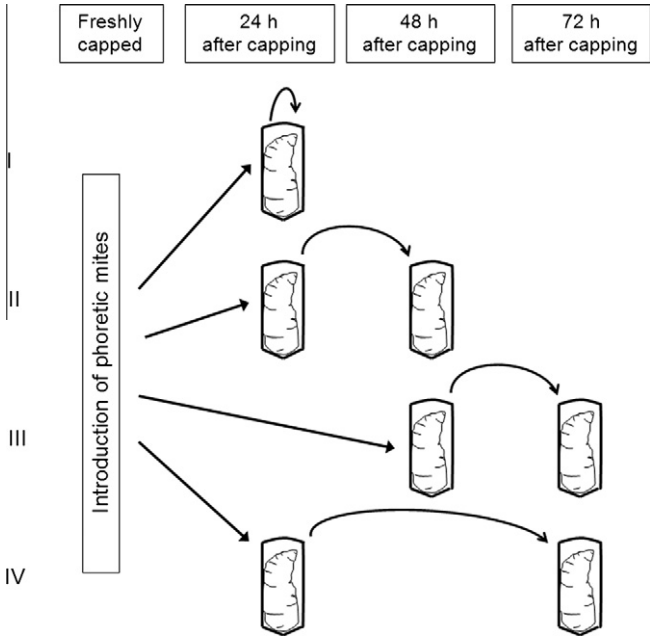

Fig. 1. Procedure of mite transfer among brood cells: phoretic mites were introduced into freshly capped brood cells and transferred after 24 and 48 h, respectively, into brood cells of different age. I (control, $n = 30$): Transfer from 24 h sealed brood cells to 24 h sealed brood cells. II ($n = 36$): Transfer from 24 h sealed brood cells to 48 h sealed brood cells. III ($n = 36$): Transfer from 48 h sealed brood cells to 72 h sealed brood cells. IV ($n = 29$): Transfer from 24 h sealed brood cells to 72 h sealed brood cells.

A subset of non-reproducing mites (n = 36) was dissected in order to prove the presence of spermatozoa in the spermatheca. The mites were dissected in PBS saline buffer by removing the dorsal shield to expose reproductive organs.

2.4. Application of 5th instar larval extracts into worker brood cells

The extraction was performed according to the method described by Garrido and Rosenkranz (2004). Briefly, non-infested and freshly capped worker larvae (5th instar, 0 h, see Section 2.2) were carefully removed from the brood cells. The larvae were first placed on a filter paper to check if hemolymph was leaking out and only non-injured larvae were used for the extraction. Groups of 10 larvae were pooled within a clean Erlenmeyer flask, filled up with 5 ml *n*-pentane (Uvasol) and extracted for 10 min at room temperature. Then the extract was removed from the larvae and concentrated with nitrogen to about 1 ml and stored at −20 °C. Before the start of the experiment the extract was further concentrated to 4 µl in order to reduce the amount of solvent for the application in the bioassay. These 4 µl represent the extract of ten larvae and, therefore, an application of 1 µl equals 2.5 larval equivalents.

To test the effect of these extracts on mite reproduction, we used marked worker brood cells which had already been sealed for 24 h (see above). This larval stage is not able to activate mite oogenesis (see results in Section 3.1, Fig. 2). We introduced phoretic *V. destructor* females into these brood cells. Before the introduction of the mites, we applied 2 µl of the larval extract (=5 larval equivalent) topically on the larvae in the opened brood cell (n = 24). To the control cells, the same amount of solvent (pentane) was applied (n = 26). After 2 min of evaporation of the solvent, the mite was introduced and the capping of the brood cell was closed. A third portion of the brood cells remained untreated (n = 30) before the introduction of the mites. All applications and controls were performed within the same colony during the same time period.

2.5. Chemical analyses

Here we focus on the quantification of cuticular methyl and ethyl esters because for these compounds a biological activity has already been confirmed (reviewed in Dillier et al. (2006)). Drone and worker larvae of five different age groups (freshly capped and 12, 24, 48, 72 h after cell capping) were extracted for 10 min in 1.5 ml *n*-pentane. These larval stages were chosen because of their different capacity to activate or inhibit the mite oogenesis in the previous experiments (see results in Sections 3.1 and 3.2). For each age group three different extracts with four worker larvae and three drone larvae, respectively, were produced. The extracts were concentrated under a purified flow of nitrogen and added with methyl tridecanoate and hexadecane as internal standards (10 ng/µl each).

The quantification of the injected extracts (2 µl/sample) was performed by GC Varian 3900 (equipped with a splitless injector (250 °C) and Varian Saturn 2100T MS SIM detector) on a capillary column (HP5-MS, length 30 m, internal diameter 0.32 mm, film thickness 25 µm). The temperature of the oven was programmed to increase from an initial setting of 50 °C (3 min) to 280 °C, 10 °C per minute. Temperature was held then at 280 °C for 11 min. Hydrogen was used as carrier gas. Three repetitions were performed for each developmental stage.

2.6. Statistical analysis

We compared the mite reproduction between differentially treated brood cells (Section 2.4) and between differentially treated *Varroa* females (Sections 2.1–2.3) with χ^2 tests on a cross contingency table and an unpaired *t*-test for the number of offspring after confirmation of normal distribution (Kolmogorov–Smirnov-test; WinSTAT Software, R.K. Fitch 2009).

3. Results

3.1. Mite reproduction after natural and artificial infestation of brood cells

The artificial infestation of brood cells with one *Varroa* female did not elicit higher removal rates in the test colonies. Overall, less than 20% of the infested brood cells were removed within the test period of about 8 days. In naturally infested brood cells, about 82% of the invaded *V. destructor* reproduced (n = 90; Fig. 2). In drone brood, this fertility rate was similar (79.4%, n = 68; Fig. 2). Artificial infestation in recently capped worker brood cells did not affect this percentage significantly (83.5%, n = 30; χ^2 = 0.019, p = 0.57; Fig. 2). However, the percentages of reproducing *V. destructor* decreased

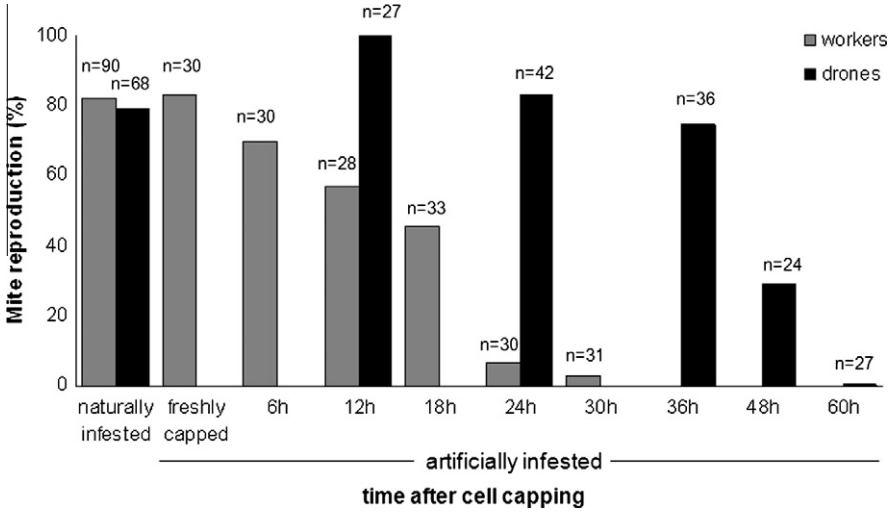

Fig. 2. Percentage of reproducing *V. destructor* females in naturally infested worker and drone brood cells (left two columns) and in brood cells which were artificially infested with phoretic mites at different times after the capping of the brood cells. With increasing time intervals between cell capping and artificial mite infestation the rate of reproducing mites decreased. In worker brood, the rate of reproducing *Varroa* females is significantly reduced already 12 h after cell capping (n = 28; χ^2 = 5.08, p < 0.05) in drone brood only 48 h after cell capping (n = 24; χ^2 = 20.05, p < 0.01).

clearly, if the mites were introduced in brood cells 18–60 h after cell capping. In worker brood, mites that were introduced 12 h after cell capping already showed a significantly lower rate of reproduction compared to natural invaded mites ($n = 28$; $\chi^2 = 4.79$, $p < 0.05$). And hardly any of the mites reproduced if they were introduced 24 h and 30 h after cell capping (Fig. 2).

In drone brood, there was only a slight decrease in the rate of reproducing mites if they were introduced in brood cells up to 36 h after cell capping (at 36 h: $n = 36$; $\chi^2 = 0.27$, $p = 0.61$). A significant decrease was only confirmed for those mites that were introduced in drone brood cells 48 h after cell capping ($n = 24$; $\chi^2 = 20.05$, $p < 0.001$). After introduction into brood cells 60 h after cell capping not a single *V. destructor* egg was laid (Fig. 2).

3.2. Reproduction of Varroa mites that have been transferred into brood cells of different ages after initiation of oogenesis

The reproduction of mites that were treated twice (introduced and transferred) was not negatively affected by the handling process: 83.3% of the mites ($n = 30$) that were artificially introduced into freshly capped brood cells and transferred 24 h postcapping to brood cells containing the same larval stage reproduced successfully with an average number of 3.4 ± 1.4 offspring per mother mite (Table 1a). This is equal to the fertility rate to naturally invaded mites (82.2%; Fig. 2).

When mites were transferred from brood cells 24 h after cell capping into cells 48 h after cell capping, the fertility decreased highly significant compared to the control ($n = 36$; $\chi^2 = 14.939$, $p < 0.001$). However, the transfer of *Varroa* mites from brood cells 24 h and 48 h after cell capping, respectively, to brood cells 72 h after cell capping did not reveal a significant effect on the mite's fertility (24–72 h: $n = 29$; $\chi^2 = 2.469$, $p = 0.116$; 48–72 h: $n = 36$; $\chi^2 = 2.377$, $p = 0.123$) albeit the number of mites with unsuccessful reproduction (egg only or male only; Table 1) increased significantly.

Compared to the control, the fecundity (=number of offspring) decreased in all experimental groups, however, only in the case of 24–72 h transfer the differences were significant (*t*-test, $p < 0.01$; Table 1).

Non-reproduction of the introduced mites was not associated with a lack of stored spermatozoa in the spermatheca. In all of the 36 non-reproducing mites from the different approaches we could verify microscopically the presence of sperms.

Table 1
Reproduction of *Varroa* mites that have been introduced into freshly capped brood cells and removed 24 h after cell capping. Mites with activated oogenesis were then transferred to brood cells of different stages. Presented are the reproduction parameters (a) and the results of χ^2-tests for differences in fertility between all groups (b).

Trial	Reproducing mites (fertility) (%)	Avg. progeny per mite (n)	Incomplete reproduction of reproducing mites		
			Egg only (%)	Daughter(s) only (%)	Male only (%)
a					
24 h → 24 h (control, $n = 30$)	83.3	3.4 ± 1.4	8.0	0	4.0
24 h → 48 h ($n = 36$)	36.1	2.5 ± 0.9	7.7	0	0
48 h → 72 h ($n = 36$)	66.7	2.5 ± 1.1	8.3	0	16.7
24 h → 72 h ($n = 29$)	65.5	2.3 ± 1.1	15.8	0	10.5

Trial	24 h → 24 h (control)	24 h → 48 h	48 h → 72 h	24 h → 72 h
b				
24 h → 24 h (control)	–	<0.001**	0.123	0.116
24 h → 48 h	–	–	0.009**	0.018*
48 h → 72 h	–	–	–	0.922
24 h → 72 h	–	–	–	–

* $p < 0.05$.
** $p < 0.01$.

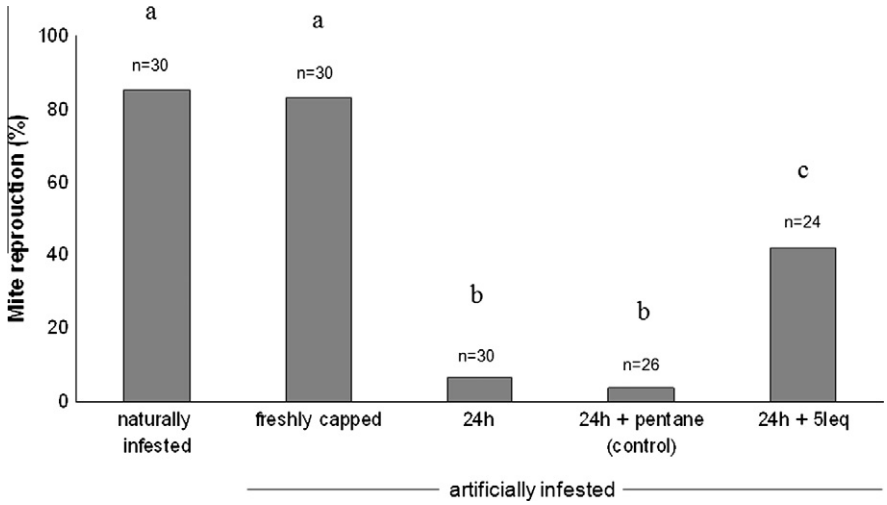

Fig. 3. Effect of the application of a solvent extract from 5th instar worker larvae (five larvae equivalents) on the activation of *V. destructor* reproduction. In naturally invaded brood cells and freshly capped brood cells, artificially infested with a single mite, between 80% and 90% of the mites reproduced. From mites which were introduced 24 h after cell capping, less than 7% reproduced, but after the application of larval extract more than 40% reproduced. Columns followed by different letters differ significantly from each other (a and b: $\chi^2 = 35.6$, $p < 0.001$; b and c: $\chi^2 = 9.2$, $p < 0.01$; a and c: $\chi^2 = 12.01$, $p < 0.01$).

3.3. Effect of the application of larval extract in worker brood cells

In an additional set of experimental setup we could at first confirm the reproduction results presented in Section 3.1 (Fig. 2): The fertility rate of natural invaded and in freshly capped brood cells introduced *V. destructor* females were 85.3% and 83.3%, respectively (Fig. 3). We also confirmed that only about 5% of the mites do reproduce if they were introduced in brood cells 24 h after the cell capping. However, if the larvae in these 24 h brood cells are treated with five larval equivalent of a pentane extract of freshly capped larvae, the percentage of reproducing *V. destructor* increased significantly ($\chi^2 = 9.2$; $p < 0.01$) to more than 40%; the application of the solvent alone had no effect (Fig. 3).

3.4. Chemical analysis of worker and drone larvae extracts

Five different fatty acid methyl esters (FAME: methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate) and three fatty acid ethyl esters (FAEE: ethyl palmitate, ethyl stearate, ethyl oleate) were quantified in the cuticular extracts from *A. mellifera* worker and drone larvae (Fig. 4). The amount of the single compounds varied among age and sex: in freshly sealed drone larvae the total amount of methyl esters were about four times higher compared to worker extracts, the total amount of ethyl esters were about two times higher (Fig. 5). Regarding the larval age, the ratio between FAME and FAEE revealed considerable differences during the first 72 h after cell capping. While at the

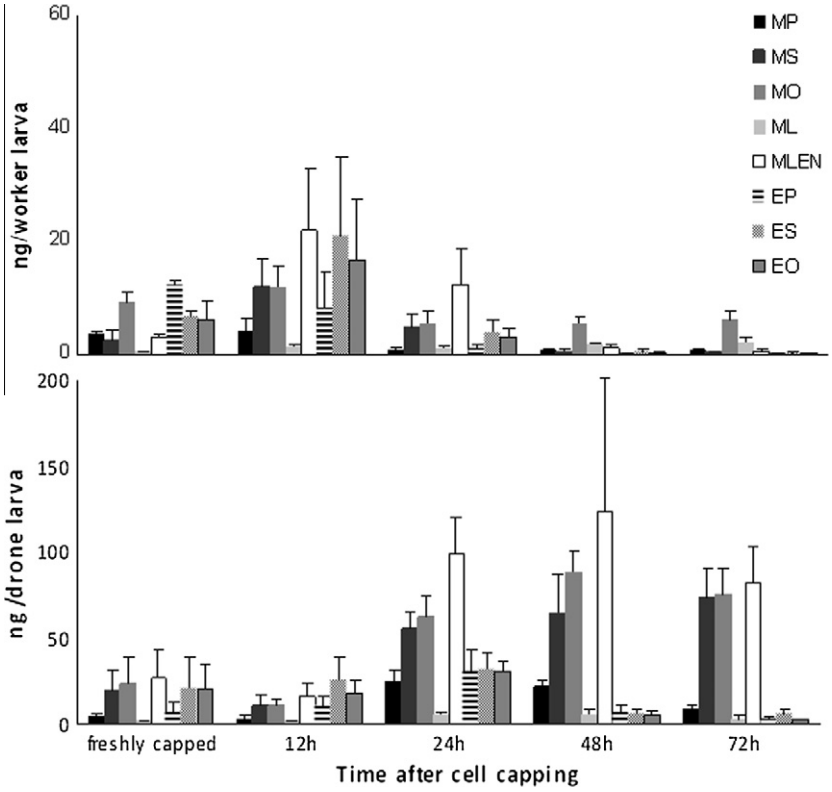

Fig. 4. Comparative amounts of the different fatty acid esters present in the cuticle of freshly capped worker and drone larvae and in larvae 12, 24, 48 and 72 h after cell capping. *MP* methyl palmitate, *MS* methyl stearate, *MO* methyl oleate, *ML* methyl linoleate, *MLEN* methyl linolenate, *EP* ethyl palmitate, *ES* ethyl stearate, *EO* ethyl oleate.

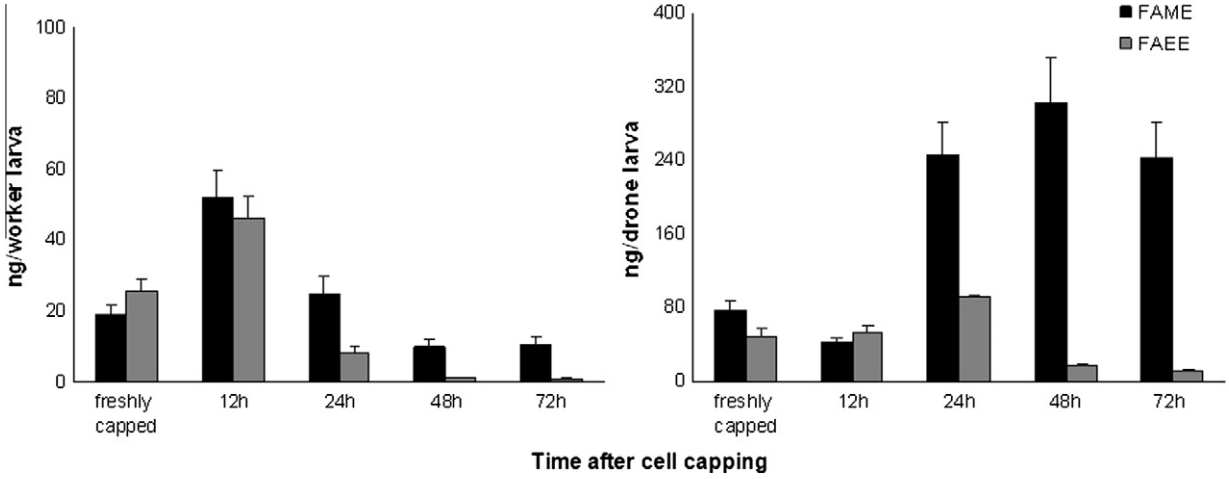

Fig. 5. Averages of total amounts ($n = 3$ for each larval stage) of FAME (Fatty Acid Methyl Ester) and FAEE (Fatty Acid Ethyl Ester) in worker (left) and drone (right) larvae within the first 3 days after capping of the brood cells.

beginning of the capping period FAME and FAEE were present in similar proportions, the amount of FAEE decreased strikingly in worker larvae 24 h and drone larvae 48 h after cell capping. In larvae 72 h after cell capping only traces of FAEE were detectable in both, worker and drone larvae (Figs. 4 and 5).

4. Discussion

The suppression of the reproductive ability of *Varroa* females by the host is still considered a crucial character to affect the mite's population dynamic (Fries et al., 1994; Rosenkranz and Engels, 1994; Correa-Marques et al., 2003; reviewed in Rosenkranz et al. (2010) and Locke and Fries (2011)). In our experiments, we could clearly show that stage specific factors of the host larva are involved in the triggering of the mite's reproduction. At first, the rate of reproducing *Varroa* females decreased significantly if they were introduced into brood cells 18 h (worker) and 48 h (drones), respectively, after cell capping. This demonstrate that only worker larvae within the first 12 h after the cell capping and drone larvae within the first 36 h after cell capping, respectively, possess the entire capacity to activate the reproduction of *V. destructor* females. Hence, we could define for the first time the exact developmental larval stages for both workers and drones that contain the crucial signals to activate the *Varroa* oogenesis.

The nature of these signal(s) still requires final clarification. There is clear evidence that polar compounds of the cuticle of freshly capped larvae are involved in the early step of activation (Garrido and Rosenkranz, 2004). This is confirmed by our application of larval extract to worker brood cells 24 h after cell capping: In untreated or solvent treated brood cells of this stage less than 5% of the introduced *Varroa* mites reproduced whereas more than 40% of the mites start reproduction after application of the larval extract. The significant lower fertility rate in the treated brood cells compared to naturally invaded mites indicates that further factors such as nutritional signals from the larval hemolymph are required to initiate and perform the complete reproductive program.

The results of this study suggest that the female mite synchronizes its reproduction with the ontogenetic development of the host larvae. From an adaptive point of view we can assume a selection pressure on an immediate start of mite oogenesis after invasion of a brood cell. For this purpose, volatiles of the host larvae could be used (Garrido and Rosenkranz, 2004) because they can be perceived even during the first hours after invasion of the brood cell when the mite is stuck to the larval food (Ifantidis et al., 1988). Under natural condition the trapped mites are released from the larval food within 1–6 h after sealing of the brood cell (Ifantidis et al., 1988). Therefore, it cannot be excluded that nutritional factors of the larval hemolymph are additionally involved in this first step of activation.

The further reproductive course of *Varroa* females is likewise influenced by the host larvae. This has already been shown for the sequence of sexes of the mite's offspring (Garrido and Rosenkranz, 2003) and for the number of offspring (Martin and Cook, 1996). With our transfer of mites between brood cells of different stage we could confirm for the first time that an already initiated oogenesis of the *Varroa* female can be interrupted by host factors. If *Varroa* females are transferred from a brood cell 24 h after cell capping – at this stage reproducing mites have already completed oogenesis resulting in a oocyte of about 300 μm (Steiner et al., 1994) – nearly 2/3 of the mites stopped their reproduction and did not lay any egg, while in the control only 17% of the mites remained infertile. Again, this makes sense from an evolutionary point of view: If the development of the oocyte is delayed compared to the development of the larvae there will be hardly a chance to finish the complete development of at least one male and one female offspring within the capping period of the brood cell. Under these conditions it might be adaptive to degrade the growing oocyte by oosorption (Steiner et al., 1995) and, therefore, save resources for the next reproductive cycle. This stop signal seems to be present in the larvae 48 h after cell capping but not to the full extent in larvae 72 h after capping. A transfer of *Varroa* females from brood cells 24–72 h after capping increased the proportion of non-reproducing *Varroa* females only slightly, however, it significantly reduced the fecundity of the transferred mites (number of offspring) and increased the proportion of mites with non-successful reproduction (i.e. egg only, male only, no male). This effect was obviously not associated with a lack of sperm: in a randomly collected proportion of about 25% of the transferred mites all dissected spermathecae were filled with sperms. This is in accordance with our previous observations (Garrido, 2004) and recent results of Kirrane et al. (2011).

Because of the transfer experiment results, we assume that oosorption leading to a temporary infertility of the *Varroa* female is only possible during the phase of oogenesis until the end of the blastoderm stage (0–48 h after cell capping; Steiner et al., 1994). At later stages a degradation of the already formed embryo may not be possible any more. This is confirmed by the results of the transfer of *Varroa* females from brood cells 48 h after capping to 72 h where we did not observe a significant reduction, neither in fertility nor in fecundity.

It is likely that both, semiochemical and nutritional signals of the host are involved in the triggering of the reproductive cycle. So far, we do not know much about the nutritional requirements of reproducing mites except the fact that proteins of the host larvae are stored directly in the ovary of the mite (Tewarson and Engels, 1982).

Considerable more data exist on the effects of stage specific cuticular volatiles of the honey bee larvae. Certain aliphatic esters play an essential role in the brood recognition by the nurse bees (Le Conte et al., 1990, 1994) and are also involved in the host finding of reproductive *Varroa* females (Le Conte et al., 1989, 1994; Trouiller et al., 1992). The secretion of these esters by the honey bee larva reveals a clear ontogenetic pattern with a maximum at the time of cell capping and a significant decrease during the following days (Trouiller et al., 1991, 1992). However, these results focus on the period before and during the cell capping and the associated invasion behavior of female *V. destructor*. A quantitative analysis of these cuticular compounds in relation to the mite's reproduction has not been published so far. We here analyzed in detail the quantitative pattern of 5 methyl and 3 ethyl esters on the larval cuticle from those larval stages that are supposed to have either an activating or inhibiting effect on the mite's reproduction. Our quantification revealed a similar pattern of FAME and FAEE within the freshly sealed larvae but lower total amounts per larvae compared to Trouiller et al. (1992) which might be due to different extraction methods. Surprisingly, the decrease of total esters within the first 2–3 days after cell capping is mainly caused by the FAEE while the amount of FAME is reduced only slightly or, in the case of drone brood, the amount is even increasing. In both worker and drone larvae, there is a striking decrease of FAEE exactly in those larval stages that are no longer able to activate the reproduction of *V. destructor*, i.e. worker larvae 24 h and drone larvae 48 h after cell capping. FAEE could therefore be involved in the first activation process for *Varroa* reproduction. This is rather speculative at the moment and requires the confirmation of a causative correlation; however, it has already been shown that certain FAME and FAEE are used by *V. destructor* as kairomone (Le Conte et al., 1989, 1994; Trouiller et al., 1992) and moreover are part of the *Varroa* female's sex pheromone (Ziegelmann et al., under review). Even though these aliphatic esters represent promising candidates it should be noted that other compounds like hydrocarbons from

the non-polar fraction of the larval cuticle could be additionally involved in the chemotactic control of mite reproduction.

So far, we do not have a reliable suggestion for the nature of the stop signal. Heptadecene is the only semiochemical which is considered to have a fecundity-reducing potential in *V. destructor* (Nazzi et al., 2002) and is obviously produced by the honey bee larvae under stress conditions. The results of our control experiments do not indicate a stress reaction of the larvae after the artificial introduction of mites so that it is unlikely that this compound is involved in the here described interruption of mite's reproduction.

Except for the suggestion of the FAEE as a potential initial activator of the mite's oogenesis, we cannot specify the signals involved in the host derived triggering of *Varroa* reproduction. For further research, an *in vitro* system allowing artificial feeding of reproducing mites is urgently needed. Since the work of Bruce et al. (1988, 1991) on artificial feeding of mites, no promising approach has been undertaken.

Our experiments confirm once again that the reproduction of the parasitic mite *V. destructor* is activated by host factors of the honey bee larva. This has already been assumed by Trouiller and Milani (1999) who stimulated the reproduction of *Varroa* within artificial gelatin cells by application of an extract of 5th instar larvae. Later on, Garrido and Rosenkranz (2004) showed that volatiles emitted by the freshly capped larvae are involved in the activation of the mite's oogenesis.

We here used a within colony approach to verify these results under natural conditions. The introduction of mature *Varroa* females into honey bee brood cells has been used successfully for many years at our lab. The introduction of phoretic mites – collected from nurse bees – into freshly sealed brood cells obviously resembles the situation of natural invaded mites. We did not record any differences in the fertility of artificial introduced and natural invaded mites. This confirms former results using this well-established technique (Rosenkranz and Stürmer, 1992; Rosenkranz and Bartalszky, 1996; Martin and Cook, 1996; Garrido and Rosenkranz, 2003) however it contradicts recent results from Kirrane et al. (2011) where nearly all of the introduced phoretic mites (and most of the transferred brood mites) remained infertile. So far, we do not have a satisfying explanation for these differences.

Our results provide an important tool for the further analysis of the crucial host signals by analyzing those host stages that have the capacity to activate or inhibit the *Varroa* reproduction. This may also help to better understand the recently confirmed genetical basis of non-reproduction in *V. destructor* (Behrens et al., 2011).

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Three QTL in the honey bee *Apis mellifera L.* suppress reproduction of the parasitic mite *Varroa destructor*

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Abstract

Varroa destructor is a highly virulent ectoparasitic mite of the honey bee *Apis mellifera* and a major cause of colony losses for global apiculture. Typically, chemical treatment is essential to control the parasite population in the honey bee colony. Nevertheless a few honey bee populations survive mite infestation without any treatment. We used one such *Varroa* mite tolerant honey bee lineage from the island of Gotland, Sweden, to identify quantitative trait loci (QTL) controlling reduced mite reproduction. We crossed a queen from this tolerant population with drones from susceptible colonies to rear hybrid queens. Two hybrid queens were used to produce a mapping population of haploid drones. We discriminated drone pupae with and without mite reproduction, and screened the genome for potential QTL using a total of 216 heterozygous microsatellite markers in a bulk segregant analysis. Subsequently, we fine mapped three candidate target regions on chromosomes 4, 7, and 9. Although the individual effect of these three QTL was found to be relatively small, the set of all three had significant impact on suppression of *V. destructor* reproduction by epistasis. Although it is in principle possible to use these loci for marker-assisted selection, the strong epistatic effects between the three loci complicate selective breeding programs with the Gotland *Varroa* tolerant honey bee stock.

Introduction

The parasitic mite *Varroa destructor* is the most dangerous parasite of the western honey bee *Apis mellifera* (Rosenkranz et al. 2010). By feeding on the hemolymph of developing and adult bees, the mite damages the bees physically and physiologically. The most devastating effects of the mite, however, are caused by its ability to vector several highly pathogenic honey bee viruses, dramatically increasing viral disease in the colony and often leading to colony death (Böcking and Genersch 2008). So far more than 18 honey bee viruses have been described and many are associated with *Varroa* mite infestation, most notably deformed wing virus (Chen and Siede 2007; Ribière et al. 2008).

The problem arose four decades ago after the mite's transition from its original host, the eastern honey bee *A. cerana* (Oldroyd 1999). The mite spread across the globe within few decades and today only Australia (Oldroyd 1999; Anderson and Trueman 2000; Rosenkranz et al. 2010), northern Sweden and Norway (SJVFS 2010), some extremely isolated populations on islands (e.g. Ile d'Ouessant: Tentcheva et al. 2004), and remote oases in deserts (Shaibi et al. 2010) have managed to remain free of *Varroa* infestations.

With the exceptions of Africanized and African bee races, apiculture with the western honey bee is nearly impossible unless regular mite control treatments (usually chemical acaricides) are used to control the parasite population (Rosenkranz et al. 2010). In temperate climates, a colony,

once it is infested with *V. destructor*, will collapse without mite control treatment within 2–3 years (Rosenkranz et al. 2010; Böcking and Genersch 2008). In the past decades, several chemicals have been used to control *V. destructor* infestations, but unfortunately the mite rapidly evolved resistance against these chemicals and their efficiency declined (Lodesani et al. 1995; Elzen and Westervelt 2004; Pettis 2004). In addition, control treatments often cause contamination of the apicultural products including acaricide residues in honey and pollen (Wallner 1999; Martel et al. 2007). It is therefore apparent that alternative strategies are needed to fight *V. destructor* that will neither facilitate resistance in the parasite populations nor contaminate bee products, thus ensuring both consumer health and customer trust in honey bee products.

In spite of the global Varroosis disaster, a few populations of European honey bees have been identified to survive infestations without any form of mite control treatment. These populations have not been managed by bee breeders but rather evolved tolerance through natural selection by mite infestation (De Jong and Soares 1997; Kefuss et al. 2004; Fries et al. 2006; Le Conte et al. 2007; Seeley 2007). *Varroa* tolerance may be based on very different traits, since the interaction between the mite and the host is very complex. A particularly well-studied behavioral trait that can lead to colony tolerance is the so-called hygienic behavior of the honey bee (Böcking and Spivak 1999). This trait is important for mite resistance of the eastern honey bee *A. cerana* (Peng et al. 1987) and has been in focus of various breeding programs in the western honey bee *A. mellifera* (Rinderer et al. 2010). Hygienic behavior has been shown to be controlled by quantitative trait loci (QTL) (Lapidge et al. 2002; Oxley et al. 2010) influencing the task thresholds for uncapping and removal of dead, diseased, or parasitized brood (Rothenbuhler 1964; Moritz 1988).

However, a more direct path toward mite resistance is the ability of the individual larva or pupa to prevent mite reproduction in the brood cell (Fries et al. 1994). The mite's reproduction is closely synchronized with that of the infested developing pupa, and different compounds of the larval cuticle are responsible for initiating egg laying by the mite (Garrido and Rosenkranz 2003, 2004).

After a decade of natural selection for survival without treatment, it has been demonstrated that mite reproductive success is reduced to about 50% in the honey bee population on the island of Gotland (Locke and Fries 2011). Cross-infestation experiments with the honey bee population on Gotland demonstrated that the observed mite tolerance in this population is a trait of the bees, and not one of the local mite population (Fries and Bommarco 2007). Mite infertility was one of the parameters influencing the reduced reproductive success of the mite in this population (Locke and Fries 2011) and is further a highly variable trait ranging between 5 and 20% in worker brood of European honey bees (Rosenkranz et al. 2010).

In this study, we aim to identify genomic regions, which influence the suppression of mite reproduction by honey bee larvae and pupae, to enable future marker-assisted breeding programs for *Varroa*-resistant honey bee stock. The availability of both the complete *A. mellifera* genome sequence (Weinstock et al. 2006) and the tolerant population on Gotland, provides an ideal setting to screen for QTL that interfere with *V. destructor* reproduction. Large sets of highly variable microsatellite markers covering the entire genome have been established (Solignac et al. 2003, 2007) and novel markers can be easily extracted from the genome sequence that can be used for high-density fine-scale mapping (Lattorff et al. 2007; Shaibi et al. 2008). In addition, because honey bees have a haplodiploid sex determination, the haploid drones provide an extremely simple and highly efficient model system for genetic studies (Moritz and Evans 2007; Moritz et al. 2010). Drones are also important for *Varroa* resistance from an epidemiological point of view, since *Varroa* mites preferentially reproduce in the drone brood of *A. mellifera* (Fuchs 1990). In the adapted host *A. cerana*, the mite reproduction is even completely restricted to the drone brood (Boot et al. 1999).

Here, we embark on using drones as a genetic model system to screen for QTL for suppression of *Varroa* mite reproduction. Because drones only have a mother queen and no father, it requires only a single generation to establish a mapping population of hundreds of individuals yielding an extremely powerful strategy for QTL identification.

Methods

Mapping population

The isolated honey bee population on the island of Gotland in Sweden has been under natural selection for mite tolerance for more than 10 years and has survived without any *Varroa* treatment (Fries et al. 2006; Locke and Fries 2011). Today, the Gotland population shows clear signs of tolerance toward *Varroa* mites and a significant reduction in the reproductive success of *Varroa* mites, whereas hygienic and grooming behavior of the bees is not increased (Locke and Fries 2011). A queen of pure Gotland origin was naturally mated to drones at the apiary of the University of Hohenheim, where the local population does not show any signs of *Varroa* tolerance or resistance and is considered to be genetically *Varroa* susceptible. Two hybrid F1 daughter queens of the Gotland queen (queen A and B in the following) were naturally mated and introduced into strong foster colonies with equally high *Varroa* infestation levels. Empty drone brood frames were added allowing the queens to produce a large drone mapping population.

Phenotypic classification

Sealed drone brood cells were opened 15–18 days after egg laying and checked for *Varroa* infestation and reproduction of the mite. Pupae infested with only a single mite with no offspring were classified either as (1) resistant ($n = 144$) and those with at least three viable offspring mites as (2) susceptible ($n = 635$). Drone pupae with intermediate reproductive success of the mite (one or two offspring mites, $n = 107$) were not included in the mapping population. This selective DNA pooling approach (Darvasi and Soller 1994) with a focus on the extreme phenotypes allows for obtaining a clear-cut segregation of individuals and alleles. After the identification of the phenotype, all drone pupae were transferred into 90% ethanol and stored at –20°C until DNA extraction.

DNA extraction and bulk segregant analysis (BSA)

Genomic DNA of all resistant ($n = 144$) and a subset of susceptible ($n = 128$) drone pupae was extracted individually from a leg, each following a modified Chelex extraction protocol (Biorad, Walsh et al. 1991). DNA concentrations were measured using the Nanodrop ND 1000 Spectrophotometer (peqlab, v 3.5.2) and equal amounts of DNA per individual were pooled according to the defined resistance phenotype from hybrid queen A (resistant, $n = 32$; susceptible, $n = 48$). We then genotyped these pools in a BSA with a total of 488 microsatellite markers distributed over all 16 chromosomes of the honey bee at 55°C following standard multiplex polymerase chain reaction (PCR) protocols (eight primer pairs per reaction; 35 cycles) (Michelmore et al. 1991; Solignac et al. 2003). Of these 488 microsatellite markers, 216 markers were heterozygous in mother queen A resulting in a resolution of one marker every 1 Mb or 19 cM on average. The mean distance between markers was 8.3 ± 0.3 cM, 78% of the genome was less than 5 cM and 96% less than 10 cM away from a heterozygous marker tested in the BSA. The marker coverage for each chromosome is illustrated in Figure S1. The obtained microsatellite fragments were analyzed with an automated DNA capillary sequencer (MegaBACE 1000) and scored with the MegaBACE Fragment Profiler Version 1.2.

For all markers, which were heterozygous in the mother queen, the fluorescence intensities (i.e., peak heights) of the two alleles (i.e., PCR products) were taken as an estimator for the allele frequencies in the DNA pools. In case of different allele frequencies between the pools (i.e., one allele predominant in one phenotypic pool, the alternative allele in the other), the ratios of the measured peak heights are expected to differ in the two PCR reactions accordingly. This difference was calculated as the sum of differences in the normalized fluorescence intensities of both alleles between the two phenotypic pools (see equation in Fig. S2) and used to select candidate regions for fine-mapping. All drones of the bulked DNA pools were then individually genotyped at these markers to confirm or reject a biased allele distribution in the phenotypic pools.

Individual genotyping and QTL-mapping

Based on the results of the BSA, all individuals from hybrid queen A were individually genotyped at a total of 131 microsatellite markers to verify the QTL candidate regions. In a single marker analysis using the software Map manager QTX (Manly et al. 2001), we identified three candidate regions, where consecutive markers showed significant different frequencies in the two phenotypic pools (χ^2-test, $P < 0.05$). We then genotyped 112 resistant and 80 susceptible individuals from hybrid sister queen B at 60 informative loci (Table S1) within these candidate regions identified in queen A to test whether the identified regions also caused a phenotypic segregation in the second mapping population. Both datasets were analyzed separately as well as pooled after reconstruction of the maternal F1 chromosomes from the haploid F2 drone offspring in both possible assignments (chromosome 1 in queen A assigned to chromosome 1 in queen B and to chromosome 2, respectively). If markers were homozygous in one of the two sister queens, these were treated as missing values in the respective part of the dataset. Pooling of datasets was done under the assumption that the resistance allele had gone to fixation in the selected and inbred Gotland population. Hence, the mother of the two sister queens is assumed to be homozygous for this allele and it must be shared by the two half-sister hybrid queens. To confirm the Gotland origin of the alleles in the resistant pool, we genotyped a pooled DNA sample of 74 drones caught at a drone congregation area on Gotland in 2007 on 40 markers in the candidate regions, and screened for common alleles to identify the maternal F1 chromosomes.

The genotypes and a binary trait value for each individual (0 for susceptible and 1 for resistant) were then entered into the software Map manager QTX (Manly et al. 2001) to calculate the suggestive and significant QTL thresholds separately for each candidate region (15,000 permutations) conducting single marker analysis and simple interval mapping. Marker positions were defined according to the genomic map Amel_4.5 (NCBI Map viewer, www.ncbi.nlm.nih.gov) and individuals were coded as double haploids. In addition, the R package R/qtl (Broman et al. 2003; R Development Core Team 2010) was used for simple interval mapping, to test for differences due to a software effect and for graphic display. Furthermore, the amount of phenotypic variance explained by each QTL separately in a single-locus model, as well as by significant epistatic interactions in a two-locus model was calculated using R/qtl. QTL regions were then screened for

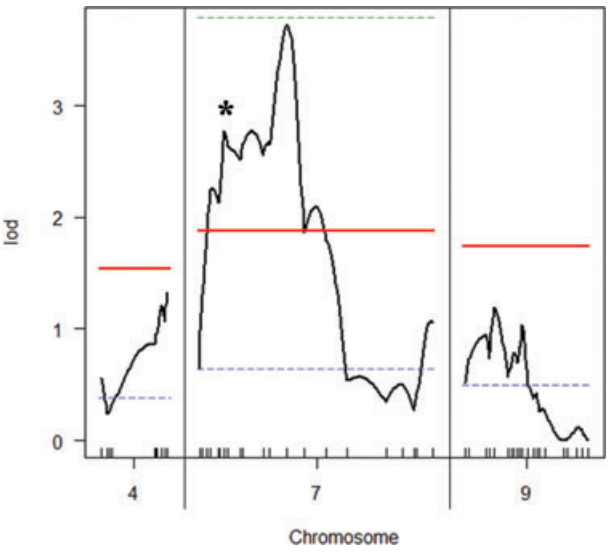

Figure 1. Candidate regions on chromosomes 4, 7, and 9 with their associated LOD scores from simple interval mapping of the pooled dataset using R/qtl. The horizontal lines represent the QTL thresholds (blue dashed: suggestive, $P < 0.63$; red solid: significant, $P < 0.05$; green dashed: highly significant, $P < 0.001$) (15,000 permutations) [Correction added after online publication 1 Nov 2011: The word 'vertical' has been replaced by 'horizontal'] . The asterisk (*) on chromosome 7 indicates the approximate position of the "futsch" ortholog (GB11509).

annotated genes in the honey bee genome database (NCBI Map Viewer; Amel_4.5).

Results

QTL candidate regions

Based on the BSA and the subsequent individual genotyping, three regions of interest, located on chromosomes 4, 7, and 9, were identified showing linkage of one or more markers in the single marker analysis to the defined trait value of host resistance. Both softwares used for simple interval mapping gave nearly identical results, indicating that the mapping results are robust, irrespective of the software applied. The results for the pooled dataset of the simple interval mapping in the three candidate regions using R/qtl are shown in Figure 1. Whereas the QTL regions on chromosome 4 (ranging from 2.1 to 4.3 Mb) and 9 (ranging from 1.0 to 3.5 Mb) were only suggestive in simple interval mapping and explained 5.3 and 3.7% of the phenotypic variance, respectively, the region on chromosome 7 (ranging from 3.6 to 8.5 Mb) significantly influenced the phenotype explaining 8.7% of the variance in a single-locus model. The majority of the designated "resistance" marker alleles in all three regions (62%, $n = 80$, Table S1) were also found in the drone sample from 2007 suggesting that our marker assignment corresponded to the alleles present in the Gotland population. This was further confirmed by the analysis of the alternative phase assignments that caused a complete loss of all QTL signals. A list of the 17 candidate genes located within a confidence interval around the highest LOD (Logarithm of the odds) score on chromosome 7 (LOD = 3.73 ± 1) is given in Table S2. The results from the simple interval mapping of the separate datasets for queen A and B are given in Figure S4.

Interactions between QTL

Using R/qtl and a two-locus model, we found a significant epistatic interaction of the QTL regions on chromosome 4 and 7 (markers UN071 and UN391). This interaction explained 3.5% of the phenotypic variance in the pooled dataset by interaction alone. In total, the two-locus model using these two markers explained 10.1% of the phenotypic variance in our dataset and was supported with a LOD score of 6.3. A heat map visualizing epistasis in the two-locus model is given in Figure S3. Figure 2 illustrates the epistatic effect on the phenotype and shows the frequency ratios between resistant and susceptible drone pupae within haplotype groups. Because we use haploid genotypes (and only because of this), it is possible to directly visualize and determine epistatic effects on the resistance trait. Drone pupae with a single resistance allele at one of the three loci did not significantly deviate from the triple susceptible haplotype. In contrast, the combination of the resistant alleles on chromosome 4 and 7 as well as the triple resistance haplotype have a more than fourfold increase in the likelihood to be resistant (two-tailed Fisher exact tests, Fig. 2). However, the interaction between the loci on chromosome 4 and 9 revealed no significant phenotypic effect and was not significant in the two-locus model. Since the individual single resistance alleles do not change the phenotype at all, but the combination of the three resistance alleles has a drastic effect, this is clear evidence of epistasis. Given the weak additive effects and the strength of epistasis, it is not surprising to see the LOD scores on chromosome 4 and 9 to be just suggestive in the individual mapping analyses.

Discussion

The suppression of mite reproduction in the pupal stage of the host seems to be under significant control by three QTL located on chromosomes 4, 7, and 9. Although the individual Gotland alleles at each identified QTL had a low effect on pupal *Varroa* resistance (Fig. 2), and two QTL were not significantly supported by simple interval mapping (Fig. 1), these loci had nevertheless highly significant impact due to their epistatic interactions. The combination of the two Gotland alleles on chromosomes 4 and 7 (47$_9$) provided almost the same suppression of mite reproduction as the combination of all three resistance alleles (479) (Fig. 2). Hence, the combination of the two QTL on chromosome 4 and 7 are of prime interest when selecting for pupal *Varroa*-resistant phenotypes in the Gotland stock. Because of the complete

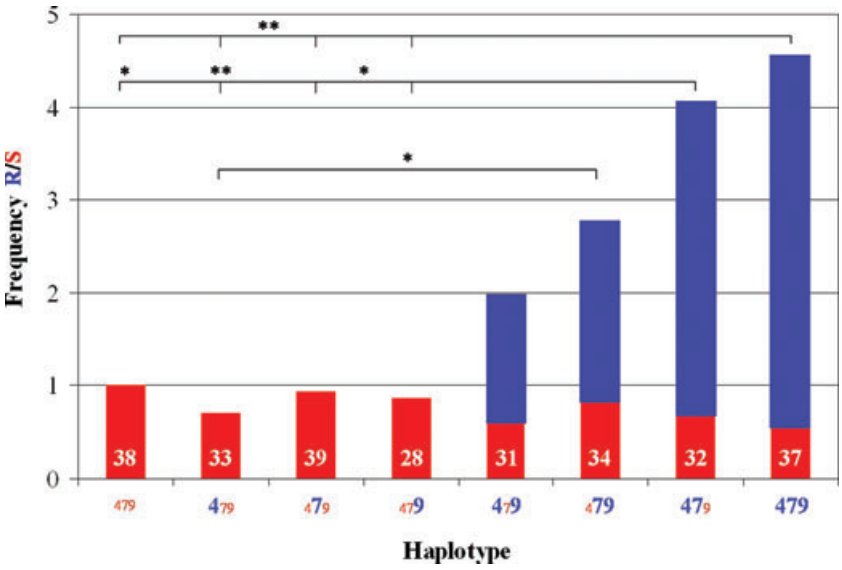

Figure 2. Frequency ratios between the number of resistant (R; $n = 144$) and susceptible (S; $n = 128$) individuals for all possible haplotypes at the three identified QTL, normalized for the frequency ratio found in the triple susceptible haplotype (479 = 1). White numbers at the bottom of the bars indicate the number of individuals with the respective haplotype. Bold blue numbers represent the marker alleles associated with the resistant phenotype, whereas small red numbers denote those alleles associated with susceptible pupae. For example, "479" represents individuals with the "resistance" marker alleles at the QTL on chromosome 4 and 7, but the alternative marker allele on chromosome 9 ($^{*}P < 0.05$; $^{**}P < 0.01$, two-tailed Fisher exact test). Blue bars illustrate the phenotypic effect of QTL interactions.

lack of additive gene effects and the strong epistatic interaction, we recommend to select for this marker combination, although this complicates marker-assisted breeding attempts. The relevant alleles responsible for this particular tolerance trait in the Gotland population are listed in Table S1. Only because we used the simple genetic make-up of haploid drones, we have been able to detect the epistatic interaction as the main driver of suppression of *Varroa* mite reproduction, which may have remained undetected in a diploid study population.

Detection limit

Before embarking on an in-depth discussion, the reader should be aware of the various general limitations and pitfalls of the QTL methodology (Slate 2005) including overestimation of QTL effects especially due to selective genotyping. The BSA in our study had an average resolution of about one heterozygous marker every 19 cM. Although major QTL are expected to produce large sweeps and should be detected even with a low density of markers, we may have missed minor QTL because of the selective genotyping approach (Darvasi and Soller 1994) and the high recombination rate of the honey bee genome. Therefore, like in any QTL study, there is a bias toward detection of major QTL versus minor QTL (Beavis 1994; Zeng 1994). In addition, the intrinsic inaccuracies of the standard bulk DNA samples analyses may not always reflect the actual genotype frequencies in the mapping populations (Michelmore et al. 1991). This can result in false positive or false negative signals and thus eventually to the nondetection of potential QTL. In spite of methodological imprecision, we are nevertheless confident to have identified three regions containing QTL involved in pupal *Varroa* resistance by suppressing mite reproduction. Clearly, we cannot exclude that additional loci that we have missed in the mapping procedure may also have been involved in the Varroosis-resistance phenotype.

Candidate genes

The identification of functional genes in the identified target regions is definitely premature for the regions on chromosomes 4 and 9. Even, the significant QTL region on chromosome 7 includes 125 annotated genes. Nevertheless, it may be worthwhile to mention two of them. One is located directly at the LOD score peak on chromosome 7, which is the ortholog of the "foxo" gene (GB11764; see Table S2), a transcription factor of the insulin signaling pathway. This conserved pathway has been assigned to diverse functions in insect growth and body size development, immune response, longevity, nutrition, cell death, and energy metabolism (Nijhout 2003a, b; Wu and Brown 2006), for example, in *Drosophila* (Jünger et al. 2003), the *Culex* mosquito (Sim and Denlinger 2008) but also in humans (Willcox et al. 2008). The foxo gene therefore appears to be a suitable candidate gene to be involved in a trait expressed during pupal development in honey bees.

The second is the ortholog to the *Drosophila* gene "futsch" (GB11509, approximate position marked with "∗" in Fig. 1). In a genome-wide expression study using microarrays, Navajas et al. (2008) found this gene to be significantly downregulated (0.86-fold) in a *Varroa*-tolerant honey bee line compared to a susceptible line. In *Drosophila*, this gene has been found to be downregulated in nonneuronal tissue during development (Hummel et al. 2000) and to be involved in phosphorylation and the induction of synaptic plasticity in

neurons. Interestingly, most differentially expressed genes between the *Varroa*-tolerant and the susceptible line in the study of Navajas et al. (2008) were involved in neuronal development and sensitivity. Although these two may be promising candidate genes for a causative relationship, we cannot exclude this as pure coincidence or provide any biological explanation at the present stage.

Impact on future breeding programs

The success of *Varroa* reproduction within the host brood cells is a crucial factor for a balanced host–parasite relationship. The most striking example is the original host, *A. cerana*, where mite reproduction is restricted to the drone brood (Boot et al. 1999) and where reproductive barriers exist between different haplotypes of the host and the parasite (Navajas et al. 2010). Suppression of reproductive success of *Varroa* females is considered as important tolerance factor in Africanized honey bees (Rosenkranz 1999) and has also been shown to be present in the European population from Gotland used in this study (Locke and Fries 2011). This suggests that selection of this trait can be achieved within the genomic architecture of the honey bee. A further advantage of the use of this trait in selection programs is that the effect on the phenotype can directly be controlled by analyzing the *Varroa* mite's reproductive success in the honey bee brood. It may therefore be highly rewarding to select for this trait in breeding programs for *Varroa* resistance. Because few genes can have major effects on this trait and individual genomes can be easily screened, marker-assisted selection (MAS) will facilitate breeding efforts more easily than for other traits that rely on complex behaviors of diploid workers (e.g., hygienic behavior). We strongly recommend taking advantage of haploid drones in mapping studies and suggest using them as a routine tool for implementing MAS in breeding programs of the honey bee. If we had not used haploid drones in this study but diploid workers instead, we very likely would have missed the significance of the strong epistatic interactions that drive the phenotype for *Varroa* resistance.

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Supporting Information

Additional Supporting Information may be found online on Wiley Online Library:

Figure S1 Marker coverage as the proportion (%) of the *Apis mellifera* genome closer than 5, 10, 20, and 25 cM, respectively, to a heterozygous marker used in the BSA for each chromosome and for the entire genome (Σ).
Figure S2 Equation used for selection of candidate regions for fine-mapping.
Figure S3 Heat map for the probability of epistatic interactions between two markers in our candidate regions for the pooled dataset.
Figure S4 Candidate regions on chromosomes 4, 7, and 9 with their associated LOD scores from simple interval mapping using R/qtl for queen A (**A**) and queen B (**B**).
Table S1. Markers used for fine mapping.
Table S2. Annotated genes in genomic region linked to the highest LOD score (LOD $= 3.73 \pm 1$) on chromosome 7.

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ORIGINAL RESEARCH ARTICLE

Invasion of *Varroa destructor* mites into mite-free honey bee colonies under the controlled conditions of a military training area

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Summary

The honey bee mite *Varroa destructor* can be spread between colonies by vertical transmission, particularly when heavily infested colonies are robbed by foraging bees from neighbouring hives. We quantified the invasion of *V. destructor* into mite free colonies on a military training area not accessible to other beekeepers. Ten "mite receiver colonies" continuously treated against *V. destructor* were placed at distances of one to 1.5 km from four heavily infested "mite donor colonies". Over a two month period from August to October, the population of bees, brood, and *V. destructor* in the donor colonies were estimated at three week intervals and the invasion of *V. destructor* into the receiver colonies was recorded every 7-12 days. During the experimental period, between 85 and 444 mites per colony were introduced into the receiver colonies. There were no significant differences in the invasion rates in relation to the distance between donor and receiver colonies. In total, 2,029 mites were found in the 10 receiver colonies, but these only correspond to 2.5% of the total mite population in the donor colonies at the start of the experiment. This means that the major part of the initial *V. destructor* population died together with the collapsed host colonies. Under natural conditions, a more benign behaviour should therefore be an adaptive strategy for *V. destructor*. From a practical perspective we could show that highly infested honey bee colonies present a substantial risk to already treated colonies up to distances of 1.5 km away.

Invasión de ácaros de *Varroa destructor* en colmenas de abejas libres de ácaros bajo condiciones controladas de una zona de entrenamiento militar

Resumen

El ácaro de la abeja de la miel *Varroa destructor* se puede trasmitir entre las colmenas por trasmisión vertical, en especial cuando las colmenas con alta infestación sufren pillaje por abejas foráneas de colmenas vecinas. Se cuantificó la invasión de los ácaros de *Varroa destructor* en colmenas libres de ácaros en una zona de entrenamiento militar no accesible a otros apicultores. Se situaron diez "colmenas receptoras del ácaro", continuamente tratadas contra la Varroosis, en distancias de uno a 1.5 km de cuatro "colmenas donantes del ácaro" my infestadas. Durante un período de dos meses, entre agosto y octubre, se estimó la población de abejas, la cría, y la *V. destructor* de las colmenas donantes en intervalos de 3 semanas y la invasión de *V. destructor* en las colmenas receptoras se registró cada 7-12 días. Durante el período experimental, se introdujeron en las colmenas receptoras entre 85 y 444 ácaros por colmena. No hubo diferencias significativas en las tasas de invasión en relación con la distancia entre las colmenas donantes y receptoras. En total, 2,029 ácaros *Varroa* fueron encontrados en las 10 colmenas receptoras. Sin embargo, estos ácaros sólo corresponden al 2.5% de la población total de ácaros en las colmenas donantes al inicio del experimento. Esto significa que la mayor parte de la población inicial de varroa murió junto con las colmenas hospedadoras colapsadas. En condiciones naturales, el comportamiento más benigno, podría ser una estrategia adaptativa de *V. destructor*. Desde una perspectiva práctica hemos podido demostrar que las colmenas de la abeja de miel con alta infestación presentan un riesgo considerable a las colmenas tratadas que se encuentran hasta una distancia de 1.5 km.

Keywords: Honey bee, *Varroa destructor*, invasion rate, vertical transmission, host-parasite relationship, treatment concept

APICULTURE AND SOCIAL INSECTS

Autumn Invasion Rates of *Varroa destructor* (Mesostigmata: Varroidae) Into Honey Bee (Hymenoptera: Apidae) Colonies and the Resulting Increase in Mite Populations

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ABSTRACT The honey bee parasite *Varroa destructor* Anderson & Trueman can disperse and invade honey bee colonies by attaching to "drifting" and "robbing" honey bees that move into nonnatal colonies. We quantified the weekly invasion rates and the subsequent mite population growth from the end of July to November 2011 in 28 honey bee colonies kept in two apiaries that had high (HBD) and low (LBD) densities of neighboring colonies. At each apiary, half (seven) of the colonies were continuously treated with acaricides to kill all *Varroa* mites and thereby determine the invasion rates. The other group of colonies was only treated before the beginning of the experiment and then left untreated to record *Varroa* population growth until a final treatment in November. The numbers of bees and brood cells of all colonies were estimated according to the Liebefeld evaluation method. The invasion rates varied among individual colonies but revealed highly significant differences between the study sites. The average invasion rate per colony over the entire 3.5-mo period ranged from 266 to 1,171 mites at the HBD site compared with only 72 to 248 mites at the LBD apiary. In the untreated colonies, the *Varroa* population reached an average final infestation in November of 2,082 mites per colony (HBD) and 340 mites per colony (LBD). All colonies survived the winter; however, the higher infested colonies lost about three times more bees compared with the lower infested colonies. Therefore, mite invasion and late-year population growth must be considered more carefully for future treatment concepts in temperate regions.

KEY WORDS honey bee, *Varroa destructor*, invasion rate, population growth, horizontal transmission

The parasitic mite *Varroa destructor* Anderson & Trueman is considered the most destructive threat of the honey bee *Apis mellifera* L. Recently it has been identified as one of the major reasons for periodical colony losses worldwide (Boecking and Genersch 2008, Brodschneider et al. 2010, Chauzat et al. 2010, Guzmán-Novoa et al. 2010). Even moderate *Varroa* infestation rates in autumn significantly increase the risk of colony losses during winter (Genersch et al. 2010). These results clearly indicate that the production of healthy and long-living winter bees (Amdam et al. 2004) is negatively affected by an infestation with *V. destructor*. Under temperate climatic conditions, long-living winter bees are produced in autumn when brood rearing is ceased for several months. These winter bees should not only survive the broodless period but also collect the first pollen and establish a new brood nest in spring.

This is already true at the level of the individual host bee where a *Varroa* infestation changes important physiological parameters of the winter bees (Amdam et al. 2004). At the colony level, the problem is intensified through an inverse population dynamic of host and parasite in late summer and autumn: although the population of bees and brood decrease substantially during this time of the year, the total number of mites increases exponentially throughout the whole period when the colony has brood (Fries et al. 1994, Calis et al. 1999, Wilkinson and Smith, 2002, DeGrandi-Hoffman and Curry 2004). This leads to continuously increasing infestation rates in the remaining brood and consequently increases damage in the emerging winter bees.

Under temperate climatic conditions, yearly treatments against the parasite are therefore indispensable to prevent damage of infested honey bee colonies. The effective control of *V. destructor* after the honey yield, but before the production of winter bees, is a crucial element of sustainable treatment concepts (Imdorf et al. 1996, Rice et al. 2004, reviewed in Rosenkranz et al. 2010).

However, there are frequent reports from beekeepers who complain of colony damage and high numbers of *Varroa* mites during winter treatment, although the recommended treatment has been performed in late summer (Le Conte et al. 2010). Such problems might

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in part be related to an insufficient efficacy of some treatments against mites that are protected within the capped bee brood cells (Sammataro et al. 2004). But, even after an effective summer treatment, there remain certain risks of colony damage, mainly caused by two factors. First, *Varroa* mites from other infested colonies that are not yet treated might invade into the treated colonies. The invasion rates seem to depend on the robbing activity (i.e., internest thievery of honey) and bee density within and around the apiary (Sakofski et al. 1990, Greatti et al. 1992, Goodwin et al. 2006, Frey et al. 2011). Secondly, the remaining and invading *Varroa* females will reproduce and might build a new parasite population that might reach the damage threshold before the wintering of the colony. There are few quantitative data concerning the interaction of *Varroa* invasion and the increase of mite population between summer treatment and wintering of the colony. Vetharaniam (2012) discussed the density effects on the mite population increase, i.e., a small *Varroa* starting population will show rapid growth rates compared with situations where *Varroa* growth rate is suppressed by high *Varroa* infestation and therefore limited availability of unparasitized brood cells.

Therefore, we quantified the mite invasion rate and the increase of *Varroa* population in nearly mite-free colonies between summer treatment and the start of the overwintering period. We made our observations in honey bee colonies at two apiaries situated in regions that had significant differences in the density of honey bee colonies. Because of the different colony densities, we assume the invasion pressure of *Varroa* mites differed between the apiaries. Furthermore, we quantified the effect of mite infestation rates in late summer on the overwintering ability of the honey bee colonies.

Materials and Methods

Study Sites. The experiment was conducted at two apiaries characterized by low and high bee densities in the southern part of the Baden state in southwest Germany, the region with the highest density of honey bee colonies in Germany (4.35 colonies per square kilometers; statistical information provided by the Baden State Beekeeping Association).

The study site with low bee density (LBD) was situated in the foothills of the Black Forest at 360 m above sea level (48° 6′1″ N, 8° 3′21″ O). Because of the mountainous structure of the region and the usually long and snowy winter, there are few permanent apiaries. After the late nectar flow in July 2011 was completed, all migrating beekeepers left the valley and only 50 nucleus colonies of one commercial beekeeper were left within the flight range (2.5 km) of our experimental colonies. These commercial colonies were treated twice with formic acid before the start of our experiment (two times 30 ml of formic acid 60% per colony, evaporated on a sponge), and therefore were considered as having a low mite infestation rate.

The study site with high bee density (HBD) was at the outskirts of the Upper Rhine Plain at 177 m above sea level (48° 12′ N, 7° 46′ O). As a result of the mild winter climate, this part of the Rhine Valley is a preferred region for overwintering of honey bee colonies and hosts a large number of local and migratory beekeepers. According to the list of registered beekeepers from the Veterinary office where all beekeepers are obliged to register and an additional survey together with the local beekeeper organization of Emmendingen, we identified >300 colonies with unknown status of *Varroa* treatment within the flight range of our experimental apiary.

Because of the abundance of *Impatiens glandulifera* Royle (an annual invasive plant in southern Germany) and several *Solidago* species, the autumn pollen supply was sufficient at both study sites. Beginning in August, all colonies were provided with additional sugar syrup for winter stores. During the experimental period, the average temperature and precipitation were recorded 2 m above the ground for both study sites once a day at a nearby weather station (Center for Agriculture and Technology, Augustenberg).

Colony Setup and Experimental Groups. We used 28 honey bee colonies of approximately the same population, headed by queens of the local Hohenheim breeding line (*Apis mellifera carnica* Pollman) and kept in Hohenheim two-story standard hives with 10 Zander frames per storey. The hives were fitted with movable sticky bottom boards protected by a wire grid of 2 mm in diameter. Without opening the hives, dead mites could be counted on the bottom boards after falling down through the wire grid. The boards were covered with an oil-soaked layer of paper towel that reliably prevents ants and earwigs from removing dead mites. To ensure equal start conditions, all colonies were treated before the experiment against *Varroa* with two highly effective acaricides: CheckMite (active ingredient: 1.36 mg coumaphos; Bayer HealthCare AG, Leverkusen, Germany) and Bayvarol (active ingredient: 4.0 mg flumethrin 90%; Bayer HealthCare AG). One strip of each acaricide was used per storey of hive with brood.

The 28 colonies were divided randomly into four groups of seven colonies each. At each of the two study sites, two groups were established. In one group at each study site, the CheckMite and Bayvarol treatments were continued from the start of the experiment on 26th July until the winter treatment in December (hereafter referred to as "treated colonies"). The two different acaricides were applied simultaneously to ensure efficacy and to help prevent the development of acaricide resistance (Rice et al. 2004). This continuous application should have killed all invading mites before they were able to enter a brood cell for reproduction. In the other group at both study sites, the acaricides were removed after 2 wk of treatment (= one sealed brood cycle) at the start of the experiment on 26th July (hereafter referred to as "nontreated colonies"). Therefore, invading mites should have been able to reproduce within these colonies. On 18th October, the nontreated colonies were again treated with CheckMite and Bayvarol for 3 wk to determine the mite invasion up to that time. At the

beginning of December, a final oxalic acid treatment (trickling Oxuvar according to the manufacturer's recommendations; Andermatt BioVet GmbH, Lörrach, Germany) was performed on all 28 colonies to remove any remaining mites.

Evaluation of Colony Development. At 3-wk intervals (nontreated colonies) and 6-wk intervals (treated colonies), the numbers of bees and brood cells of all colonies were estimated according to the Liebefeld method (Imdorf et al. 1987). In February 2012, the number of bees of all 28 colonies was estimated for the final time to identify the loss of the adult bee population over winter.

Two combs each from the colonies of the treated and nontreated groups were removed in September and analyzed for residues of coumaphos (LOQ 0.5 mg/kg) and flumethrin (LOQ 1 mg/kg) in the beeswax according to the analytical method developed at our residue laboratory (accredited according to DIN EN ISO/IEC 17025).

Evaluation of Invasion and Infestation Rates of *V. destructor*. In the treated colonies, the invading mites that were killed by the acaricides were counted on the sticky bottom boards once a week from 26th July until 5th November. The expected mite-free status of these colonies was confirmed during the experiment by analyzing bee and brood samples taken every 6 wk. We quantified *Varroa* infestation by counting the adult female mites.

To estimate the growth of the *Varroa* infestation within the nontreated colonies, samples of adult worker bees (≈150 bees per colony) and sealed brood (≈200 worker brood cells containing pink-eyed pupae) were analyzed at 3-wk intervals during the experiment. The final mite infestation was determined according to the number of mites killed by the treatments in October and December (see above).

Data Analysis. The nonparametric Kruskal–Wallis test was used to compare bee and brood populations between groups at the start of the experiment. A repeated-measures analysis of variance (ANOVA) was used to compare the amount of brood in the nontreated colonies between the LBD and HBD sites over the entire experimental period; sites was the independent factor in the ANOVA. The Mann–Whitney *U* test was used for the comparisons of the weekly mite invasion rates in the treated groups. In October, the *Varroa* infestation levels of the nontreated colonies revealed large differences between the apiary sites. For the analysis of the decline of the bee population over winter (bee populations in October vs. bee populations in February), we used one-way ANOVA with repeated measures for the LBD and HBD sites separately. All tests were performed with the SPSS 20.0 statistics software.

Results

Climatic Conditions at Both Study Sites. The course of the average daily temperature did not differ between the areas with HBD and LBD, suggesting similar foraging conditions at both apiaries. The 2011 season was characterized by warm and stable weather conditions until mid-October followed by a short cold spell and a warm autumn. During the 100-d experimental period, on Day 92 (LBD) and Day 89 (HBD) the maximum daytime temperatures exceeded 12°C, which is considered the minimum threshold value for honey bee flight activity. On only Days 5 (LBD) and 2 (HBD) did the precipitation exceed 0.5 mm per hour, which might have prevented the bees from leaving their hives.

Colony Development. At the start of the experiment, the four experimental groups were homogenous in number of bees ($P = 0.45$; Kruskal–Wallis) and brood ($P = 0.09$; Kruskal–Wallis) with average bee populations ranging from ≈17,000–22,000 for the different groups. By the end of the season and the start of the wintering period in October, the average adult bee populations decreased in all groups from ≈20,000 bees per colony to 10,000–14,000 bees per colony. Of the 28 experimental colonies, 25 had adult bee populations of >8,000 bees in October and only three colonies had populations between 7,000 and 8,000 bees per colony. Honey bee colonies with >8,000 bees in October are considered well-prepared for successful overwintering in temperate climates (Imdorf et al. 2008).

After the start of the experiment, the brood production was significantly greater at the HBD site than at the LBD site ($F = 23.2$; $P < 0.01$; repeated measures ANOVA), probably due to a more varied range of pollen sources (Fig. 1). Overall averages of the number of brood cells in the nontreated colonies were 109,000 ± 4,471 brood cells per colony at the HBD site and 75,000 ± 3,635 brood cells at the LBD site.

***V. destructor* Invasion Rates.** The number of mites invading the treated colonies differed between the LBD and HBD sites (Fig. 2). During the entire experimental period, with the exception of the calendar Weeks 30, 31, and 33, the invasion rates into the HBD colonies were significantly higher compared with the LBD colonies ($P < 0.05$ for calendar Weeks 32 and 34 and $P < 0.01$ for calendar Weeks 35–44; *U* test). At the HBD apiary, a striking increase of the invasion rate was recorded after calendar Week 33, while at the LBD apiary, it remained continuously low over the whole period (Fig. 2).

The average invasion rates over the entire 3.5-mo period were 462 ± 74 mites per colony with a range between 266 and 1,171 mites at the HBD apiary and 126 ± 16 mites per colony with a range between 72 and 248 mites per colony at the LBD apiary. The maximum number of invaded mites per colony in a week was 109 for HBD (calendar Week 35) and 47 for LBD (calendar Week 30). For all seven colonies at each of the two apiaries, these numbers add up to totals of 3,238 introduced mites at the HBD apiary compared with only 880 mites at the LBD apiary.

The analysis of bee and brood samples of the treated colonies (taken three times during the experimental period in July, September, and October) confirmed the efficacy of the applied acaricides. We found only

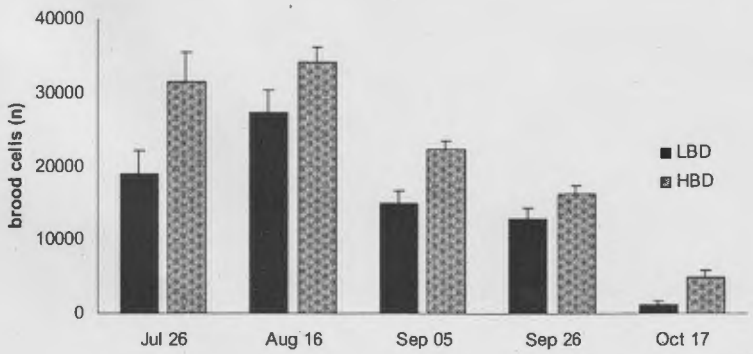

Fig. 1. Average number of worker brood cells of the nontreated colonies at study sites with LBD and HBD (means ± SE). In October, two of the LBD colonies were without brood. The differences in brood production were significant between the two sites ($P < 0.01$; repeated measures ANOVA).

eight mites in 5 of the 42 analyzed bee samples (corresponding to 8,334 analyzed bees), and only 30 mites in 15 of the 41 examined brood samples (corresponding to 7,606 analyzed worker brood cells).

***V. destructor* Infestation Rates of the Nontreated Colonies.** The average *V. destructor* infestation rate of adult bees at the LBD apiary increased from 0.0% at the start of the experiment to 4.3 ± 1.1% in mid-October (Table 1). The brood infestation rate increased from 1.1% in July to 16.0 ± 3.8% in October (Table 1). The colonies at the HBD apiary started with an average mite infestation on adult bees of 0.2% and reached a final bee infestation of 18.0 ± 3.7% 3 mo later. The brood infestation rate of this group revealed an extreme increase from 0.7% in July to 50.8 ± 10.5% in October (Table 1).

The colonies of the nontreated groups received *Varroa* treatments with CheckMite and Bayvarol in October followed by a final oxalic acid treatment in December. As a result of mite invasion and subsequent reproduction of those mites, the *Varroa* populations in these nontreated colonies reached average final infestation levels of 864–6,028 mites per colony at the HBD apiary (on average 2,028 mites per colony) but only 190–488 mites per colony at the LBD apiary (on average 340 mites per colony; Table 2). Thus, the numbers of *Varroa* mites per apiary ($n = 7$ colonies each) added up to 14,577 (HBD) and 2,380 (LBD), respectively.

The final oxalic acid treatment of the treated colonies confirmed the efficacy of the combined Bayvarol and CheckMite treatment: very low numbers of mites (3, 3, and 5) recorded in only 3 of the 14 colonies.

In the beeswax of the treated colonies, residues of coumaphos were detected in low concentrations (6.6 and 7.5 mg/kg), whereas flumethrin was not detected. In the samples of the nontreated colonies none of these active ingredients were found, indicating that the possibility of a residual effect from the previous treatment was rather low.

***Varroa* Infestation and Overwintering.** At the HBD site there was a significant difference between the treated and nontreated colony groups in the percentage loss of the adult bee population during the wintering period from October 2011 until the end of February 2012 ($F = 91.7$, df = 1, $P < 0.001$). The nontreated, highly mite infested colonies lost 58.1% of their bee population, while the treated colonies with fewer mites lost only 24% (Fig. 3). However, at the LBD site the decline of the bee population was similar and not significantly different ($F = 2.3$, df = 1, $P = 0.16$) between the moderately infested nontreated group (36.0%, on average) and the treated group (39.8%, on average).

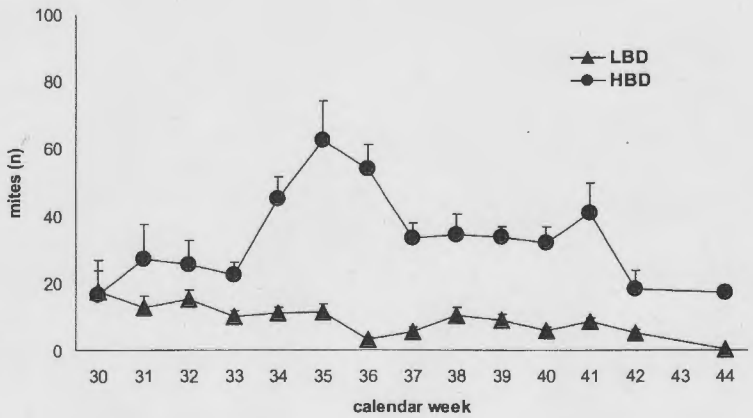

Fig. 2. Average number of *V. destructor* that invaded treated colonies at the LBD ($n = 7$) apiary and HBD ($n = 7$) apiary from the end of July until the beginning of November (means ± SE).

Table 1. Average *V. destructor* infestation rates (mites per 100 bees; mean ± SE) in sealed brood cells and on adult bees of the nontreated colonies at the LBD (n = 7) apiary and the HBD (n = 7) apiary

Sampling date	LBD		HBD	
	Bees	Brood	Bees	Brood
26 July	0.0 ± 0.0	1.1 ± 0.1[a]	0.2 ± 0.8	0.7 ± 0.4
16 Aug.	0.5 ± 0.2	0.9 ± 0.4[a]	0.9 ± 0.5	0.8 ± 0.3
05 Sept.	0.5 ± 0.1	2.6 ± 0.3	1.3 ± 0.9	6.5 ± 1.6
26 Sept.	1.2 ± 0.3	8.5 ± 1.1	6.5 ± 2.0	22.1 ± 8.8
17 Oct.	4.3 ± 1.1	16.0 ± 3.8[b]	18.0 ± 3.7	50.8 ± 10.5

[a] Brood samples taken from n = 6 colonies.
[b] Brood samples taken from n = 3 colonies.

Discussion

Under temperate climatic conditions, *Varroa* treatments have to be performed before the production of long-lived winter bees. Worker bees parasitized during development have a reduced life span (Amdam et al. 2004) and will presumably not survive until spring. In addition, a high *Varroa* infestation in the colony leads to a higher transmission of viruses among the bees (Francis et al. 2013). The close correlation between mite infestation in late autumn and winter mortality of honey bee colonies has also been confirmed by a large German bee monitoring project (Genersch et al. 2010). An effective *Varroa* control in late summer is crucial for the successful overwintering of honey bee colonies when mite infestations are threatening, and therefore is an indispensable part of an integrated pest management (IPM; Delaplane 2011, Dietemann et al. 2012).

However, the time window available for these late summer treatments is rather narrow because chemical treatments can only be started when the last honey harvest is completed (Currie and Gatien 2006). Our study indicates that the horizontal transmission of *Varroa* mites could additionally jeopardize the IPM performed by the beekeepers. We used two neighboring study sites to quantify the invasion rates of *Varroa* mites in relation to the density of honey bee colonies. The two experimental apiaries were only 21 km apart and provided nearly identical conditions in terms of ambient temperature and rainfall. At both apiaries, the provision with nectar and pollen throughout the experimental period was sufficient to stimulate brood production and prevent robbing. The crucial difference between both apiaries was the number of honey bee colonies within the foraging range. The LBD apiary was situated within an isolated valley with a low number of treated nucleus colonies in the proximity. The HBD apiary was located within a region preferred by beekeepers for overwintering their hives and represents a region with one of the highest density of honey bee colonies in Germany. Therefore, we assume a substantially higher invasion pressure of *Varroa* mites at the HBD site than at the LBD site. For the quantification of the weekly invasion rates into the experimental colonies, we used a continuous treatment with two different acaricides. The repeated sampling of adult bees and brood for *Varroa* and the nearly mite-free status of these colonies at the end of the experiment confirmed that *Varroa* mites were killed immediately after invading these colonies.

Over the entire 3.5-mo period, we recorded a total mite invasion rate of >3,200 mites into the seven treated colonies at the HBD site. This was a nearly fourfold higher number compared with the LBD apiary, although significant site-specific differences in the weekly invasion rates were identified only after mid-August. The latter confirms earlier studies assuming that *Varroa* invasion is triggered by cessation of the nectar flow in late summer and the subsequent increase in robbing among honey bee colonies (Sakofski et al. 1990, Greatti et al. 1992, Frey et al. 2011). However, our weekly invasion rates were clearly lower compared with the >30 mites per day per colony described from Italy ≈20 yr ago (Greatti et al. 1992). This difference might be explained by the fact that, in our research area, there were no indications of feral bee colonies and that, during our experimental period, there were no reports of collapsing colonies. Furthermore, because of intensive extension services and the well-organized beekeeping association in this state, local beekeepers are very much aware of the need for late summer treatments and the vast majority try to follow these recommendations.

Despite these good advisory services and long-term experience of beekeepers in treating *Varroa*, we still recorded a dangerously high invasion pressure at the HBD site from mid-August through mid-October. This leads to reinvasion of mites in previously treated colonies as is demonstrated by our largely mite-free experimental colonies. One can assume that the invading *Varroa* mites come from more highly infested colonies of neighboring apiaries. Invasion, coupled with subsequent reproduction by invading mites, can be a substantial problem for beekeepers who treated their colonies earlier, giving them a false sense of security. It also might explain, at least in part, unexpected winter mortality of colonies belonging to experienced beekeepers who have performed *Varroa* treatments according to recommendations (Le Conte et al. 2010).

At both our study sites, half of the colonies were only treated before the start of the experiment, and thereafter left untreated until a final treatment in

Table 2. Final mite infestation of the non-treated colonies at the LBD apiary and the HBD apiary in November resulting from *Varroa* invasion and subsequent reproduction during the experimental period

	LBD							HBD						
Colony no.	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Mites (n)	342	319	488	449	292	300	190	933	1,412	1,846	1,232	864	2,262	6,028

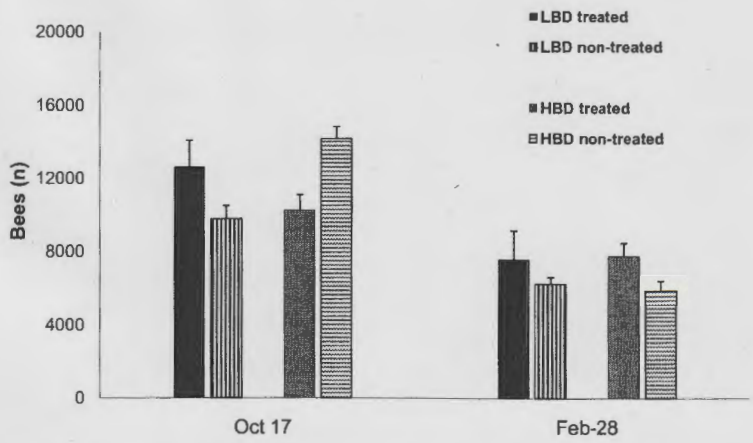

Fig. 3. Number of bees of the treated and nontreated colonies at the apiaries with LBD (moderate *Varroa* infestation) and HBD (high *Varroa* infestation) before and after overwintering (means ± SE).

November. Invaded mites could therefore reproduce over the entire 3.5-mo period. Because all experimental colonies had the same genetic background and were of similar size, we assume invasion rates in the nontreated colonies were similar to those in the continuously treated colonies in the same apiary.

There were large differences between the HBD and LBD sites for infestations of both brood and bees. At the HBD site, the average brood infestation exceeded 6% at the beginning of September. At the end of September, the average infestation rate exceeded 22%, meaning that a quarter of the hatching winter bees were already weakened. At the HBD site, the infestation level of the adult bees was comparatively low until mid-September but then clearly exceeded the autumn economic threshold of 3–5 mites per 100 adult bees suggested for the United States and Canada (Delaplane and Hood 1999, Strange and Sheppard 2001, Currie and Gatien 2006). At the LBD site, both the bee and brood infestations remained consistently below the damage threshold. However, at the last examination in October, the bee infestation was only slightly lower than the threshold level of 6 mites per 100 bees determined by the long-term monitoring of winter losses in Germany (Genersch et al. 2010).

The site-specific differences in the final infestation, as determined by treatments starting at the beginning of November and performed with three different acaricidal compounds (flumethrin, coumaphos, and oxalic acid), were large. In the colonies at the HBD site, we recorded between 933 and 6,028 mites per colony, whereas at the LBD site, we found a clearly lower range of 190 and 488 mites per colony. These values demonstrate the large variation in the infestation levels of colonies kept under identical beekeeping management conditions. In addition, it demonstrates the significant reproductive capacity of *Varroa* mites in autumn when environmental conditions allow brood rearing within the colonies. An exact calculation of the rate of *Varroa* reproduction in our colonies is not possible because invasion rates and subsequent reproduction cannot be separated within the same colony. However, if we compare the absolute number of invading mites with the average final infestation, we see a large difference at both study sites: 126 versus 340 mites at the LBD and 462 versus 2,088 at the HBD site, indicating a substantial multiplication of the invading mites. The ratios between final infestation and invading mites (2.7 at the LBD and 4.5 at the HBD site) indicate that at the HBD site the mites invaded earlier, resulting in more reproductive cycles, or that these colonies provided better conditions for *Varroa* reproduction. As the availability of brood is a crucial factor for *Varroa* population growth (Wilkinson and Smith 2002, Vetharaniam 2012), a higher reproductive rate could at least partly be explained by the higher brood production of the HBD colonies during the experimental period. Daily mite population growth rates between 0.01 and 0.025 are suggested by models (Martin, 1998, Wilkinson and Smith, 2002). Such growth rates could explain our final infestation levels if we use the data on mite invasion from our experiment within a simple exponential model of *Varroa* reproduction.

In terms of relevance for beekeeping practice, we could demonstrate that effective *Varroa* treatment at the end of July, when undertaken alone, is not sufficient for successful overwintering if the mite invasion pressure is high. It is likely that a high density of *Varroa* infested honey bee colonies within flight range will increase the invasion pressure. However, other factors like ineffective *Varroa* treatments might also influence the invasion rates independently from the colony density. Even colonies that are largely mite-free at the beginning of August can build up threatening *Varroa* populations by the beginning of winter. Our data on overwintering also emphasize the risk of high *Varroa* infestations late in the year. At the LBD apiary, where both experimental groups were either noninfested (continuously treated) or moderate infested, the average decline of the bee population from October till February was <40%. Such values are within the range reported from overwintering colonies in temperate regions (Free and Racey 1968, Imdorf et al. 2008). However, at the HBD site the heavily infested colonies lost, on average, nearly 60% of their bees, which represents a highly significant difference compared with the noninfested colonies at the same apiary. The reason that none of these highly infested colonies com-

pletely collapsed overwinter might be due to the high number of bees in October (>14,000, on average) and the fact that the *Varroa* population increased at a time of the year when a proportion of winter bees has already been produced. According to Mattila et al. (2001), the first winter bees appear at the end of August. At that time, none of the experimental colonies had a high *Varroa* infestation. Van Dooremalen et al. (2012) showed clearly that *Varroa* infested bees had a shorted lifespan and that successful overwintering depends strongly on the proportion of noninfested winter bees. Infested bees have a higher probability of being infected with bee viruses (Nguyen et al. 2011, Francis et al. 2013), which may have additionally contributed to the weakening of our colonies. Comparative virus analysis on bees from the different experimental groups before and after overwintering supports this assumption (McMahon et al. 2014).

We assume that the situation within our research sites largely corresponds to other regions with a temperate climate and intensive beekeeping activities. Because of anticipated changes in climate leading to higher autumn and winter temperatures in temperate regions (Linderholm 2006), we will increasingly be faced with conditions that support reinvasion into colonies of *Varroa* mites in autumn and their reproduction therein. Therefore, our study points out some general aspects that should be considered for the implementation of *Varroa* treatment concepts. First, IPM programs should be coordinated region-wide to reduce the *Varroa* reinvasion pressure. Second, additional *Varroa* diagnostic measures are recommended during the period after summer *Varroa* treatment. This is the only way for the beekeeper to detect and then react to unexpectedly high mite infestations.

Beside these practical recommendations, our results also point to a conflict between beekeeping practice and the selection of *Varroa* resistant honey bees. For many selection programs, colonies should be allowed to host a number of mites sufficient to demonstrate the capacity of the colony to control the growth of the mite population (Büchler et al. 2010). Therefore, *Varroa* treatments should not be performed too early and should depend on colony infestation levels. At least in regions with high bee densities, this will significantly increase the number of mites within the region and most likely, as a consequence, the *Varroa* reinvasion pressure and horizontal transmission of *Varroa* mites between colonies. Horizontal transmission of a pathogen is assumed to favor the development of a virulent host–parasite relationship (Fries and Camazine 2001). That is, *Varroa* mites that harm or even kill their colony have a realistic chance to find a new host colony for further reproduction. Hence, high bee densities combined with ineffective treatment will not only increase the risk of colony damage but might also select for more virulent *Varroa* mites.

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3 Allgemeine Diskussion und Schlussfolgerungen

Varroa destructor ist durch die weltweite Verbreitung zu einem ubiquitären Parasiten bei Honigbienen (*Apis mellifera*) geworden. Eine „Eliminierung“ des Parasiten erscheint selbst auf regionaler Ebene aussichtslos (Dietemann et al., 2012). Aus imkerlicher Sicht muss es daher vorrangiges Ziel sein, die Schäden beim Honigbienenwirt unterhalb der wirtschaftlichen Schadensschwelle zu halten. Dies setzt voraus, dass die während der Saison meist exponentiell anwachsende Varroapopulation im Bienenvolk kontrolliert wird (Rosenkranz et al., 2010). Diese Zunahme der Parasitenpopulation hängt von mehreren Faktoren ab, wobei es hier komplexe Wechselwirkungen zwischen Parasit und Wirt sowohl auf der individuellen als auch der Bienenvolk- bzw. Populationsebene gibt. In der vorliegenden Arbeit wurden einige dieser Wirt-Parasit-Wechselwirkungen genauer untersucht und dabei auch neue Aspekte bezüglich des Wirtseinflusses auf die Reproduktion der Milbe nachgewiesen.

Zunächst konnte auf der individuellen Ebene der Varroareproduktion der bereits von Garrido und Rosenkranz (2003, 2004) beschriebene Einfluss der Bienenlarve auf Aktivierung und Verlauf der Varroareproduktion konkretisiert und erweitert werden. In einem neuen *in vivo* Ansatz unter kontrollierten Bedingungen wurden Varroaweibchen in Brutzellen unterschiedlichen Alters eingesetzt bzw. nach bestimmten Zeiten wieder in andere Brutzellen umgesetzt. Dadurch konnte der Effekt unterschiedlicher Larven- und Puppenstadien auf die Varroareproduktion quantifiziert und erstmals klar gezeigt werden, dass ausschließlich frisch verdeckelte Bienenlarven in der Lage sind, die Varroaoogenese zu aktivieren (Frey et al., 2013). Bereits wenige Stunden nach Verdeckelung der Brutzelle kann die Varroaoogenese nicht mehr aktiviert werden, wobei diese Phase bei Drohnenlarven länger dauert als bei Arbeiterinnenlarven. Damit besteht erstmals die Möglichkeit, durch Vergleich der unterschiedlichen Wirtsstadien gezielt nach den Signalen zu suchen, die die Varroareproduktion aktivieren. Erste Ansätze hierzu wurden in dieser Arbeit bereits durchgeführt: Als bisher einziger konkreter Parameter von Seiten des Wirtes für die Stimulierung bzw. Hemmung der

Varroareproduktion wurden kutikuläre Duftstoffe der Bienenlarve diskutiert, die überwiegend zum Zeitpunkt der Zellverdeckelung gebildet werden (Garrido and Rosenkranz, 2004). Die biologische Aktivität dieser Substanzen konnte erstmals durch die Applikation von Kutikulaextrakten der entsprechenden Bienenlarvenstadien bestätigt werden. Wir vermuten, dass es sich bei den gesuchten Substanzen um Verbindungen aus der polaren Fraktion der volatilen Larvenduftstoffe handelt. Unsere qualitativen und quantitativen GC-MS-Analysen von Larvenextrakten zeigen eine relativ hohe Variation bei den polaren Fettsäuremethyl- und -ethylestern. So nimmt die Menge an Ethylestern bei Arbeiterinnen- und Drohnenlarven exakt bei den Larvenstadien deutlich ab, die keine aktivierende Wirkung mehr auf die Varroaoogenese haben. Diese könnten damit als Wirtssignale am ersten Aktivierungsprozess der Varroaoogenese beteiligt sein. Fettsäureester spielen somit nicht nur bei der Wirtsfindung der Varroamilbe eine wichtige Rolle (Le Conte et al., 1989; Dillier et al., 2006), sondern sind möglicherweise auch in die Reproduktionssteuerung involviert. Diese Hypothese muss allerdings noch durch weitere kausale Versuchsansätze überprüft werden, wie z.B. durch gezielte Applikationen solcher Substanzen und Substanzgemische in unserem Testsystem.

Noch spannender ist die hier erstmals nachgewiesene Existenz eines „Stopp-Signals“, das eine bereits begonnene Varroaoogenese offensichtlich wieder unterbrechen kann. Vereinfacht ausgedrückt bedeutet dies, dass ein Varroaweibchen versucht, den Verlauf seiner Oogenese mit der Ontogenese der Wirtslarve zu synchronisieren. Wenn die Oogenese im Vergleich zur Entwicklung der Larve zu spät beginnt bzw. zu langsam verläuft, wird die Oogenese wieder gestoppt. Dies macht aus evolutiver Sicht Sinn, da eine erfolgreiche Varroareproduktion voraussetzt, dass sich mindestens ein männliches und ein weibliches Ei bis zum Adultstadium entwickeln und noch vor dem Schlupf der Bienen die Begattung der Milbennachkommen stattfinden kann (Rosenkranz et al., 2010). Wenn die Muttermilbe „feststellt“, dass dies aufgrund verzögerten Reproduktionsverlaufes nicht möglich ist, können durch den Abbruch der Oogenese Ressourcen für den nächsten Reproduktionszyklus geschont werden. Dieses „Stopp-Signal“ scheint vor allem in den seit 48 Stunden verdeckelten Larvenstadien vorhanden

zu sein (siehe unten stehende Abbildung, online als „graphical abstract“ in Frey et al., 2013 enthalten). Für die chemische Natur dieses Signals liefern die bisherigen Duftstoffanalysen allerdings noch keine Hinweise.

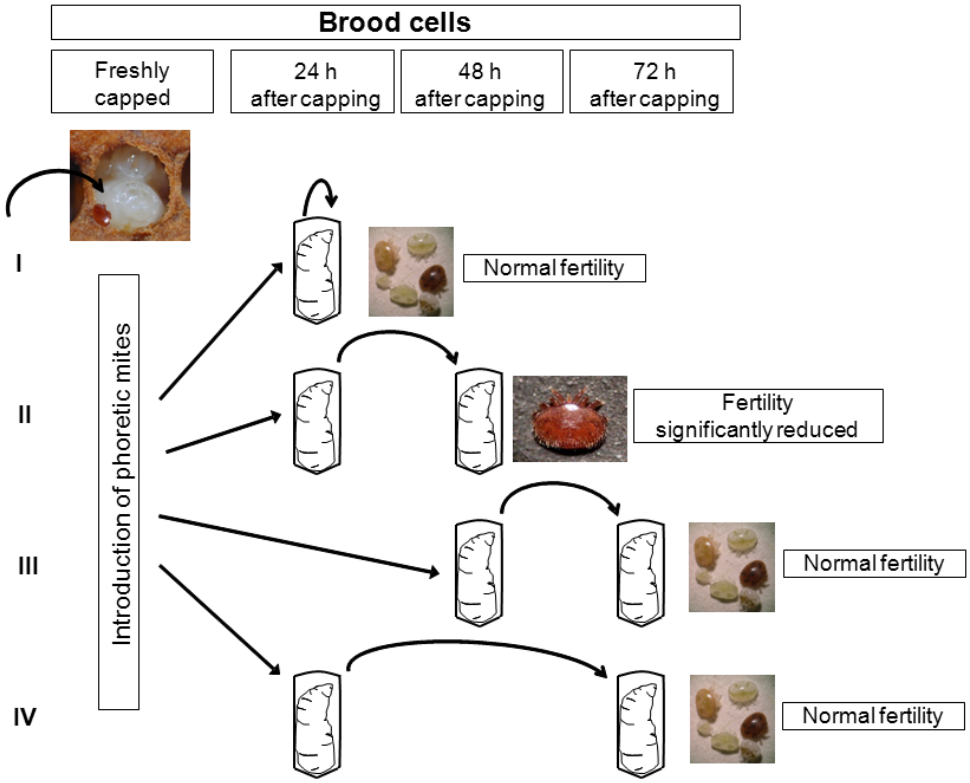

Abb. 1: Umsetzexperimente von Varroamilben zwischen Brutzellen unterschiedlichen Alters, die zeigen, dass Varroaweibchen ihre Reproduktion unterbrechen, wenn sie vorzeitig in ein älteres Larvenstadium umgesetzt werden.

Unabhängig von der Analyse der physiologischen Details bzgl. der Steuerung der Varroareproduktion ist es eine wichtige grundsätzliche Frage, ob es eine genetische Grundlage des Bienenwirtes für die Beeinflussung der Varroafertilität gibt. Als Merkmal verwendeten wir hierfür die „temporäre Infertilität“ der Varroaweibchen: Ein Teil der in die Brut eingedrungenen Varroaweibchen legt keine Eier, obwohl sie dazu in der Lage sind und in folgenden Brutzyklen auch häufig wieder erfolgreich

reproduzieren (Rosenkranz and Engels, 1994; Correa-Marques et al., 2003). Auf der Ebene der Einzelbiene wurde nun der genetische Hintergrund dieses möglichen Resistenzparameters gegenüber der Varroamilbe analysiert. Hierbei bietet die haplo-diploide Geschlechtsbestimmung bei Honigbienen besondere Möglichkeiten durch die Verwendung haploider Drohnen, da Resistenzgene eher im Phänotyp sichtbar werden und sich das Genom leichter genetisch charakterisieren lässt. Hierfür wurden Hybridköniginnen aus der vorselektierten „Gotland-Population“, die seit über zehn Jahren ohne Varroabekämpfung überlebt hatte, mit unserer nicht auf Varroaresistenz selektierten Hohenheimer Zuchtlinie erstellt. Dabei musste beachtet werden, dass aufgrund der haplo-diploiden Geschlechtsbestimmung bei Honigbienen die entsprechenden männlichen F1-Phänotypen erst in der nächsten F2-Generation auftreten. Die Phänotypisierung der Drohnen für die anschließende genetische Analyse erfolgte anhand der Fertilität bzw. Infertilität der Varroaweibchen in individuellen Drohnenbrutzellen. Die anschließende molekulargenetische Feinkartierung der Drohnen zeigte, dass die Unterdrückung der Milbenreproduktion (= Infertilität) mit drei QTL (Quantitative trait loci) auf den Chromosomen 4, 7 und 9 der untersuchten Wirts-DNA korreliert ist.

Zwar haben die Allele an den jeweiligen QTL einzeln betrachtet nur einen relativ geringen Effekt, doch haben die drei Loci zusammen aufgrund der epistatischen Wechselwirkungen einen hochsignifikanten Effekt auf die Varroareproduktion. Interessanterweise scheint es bei einem QTL eine Orthologie zum “foxo”-Gen zu geben, dem Funktionen im Zusammenhang mit Immunantwort, Langlebigkeit, Zelltod und Energiemetabolismus zugeschrieben werden (Nijhout, 2003; Wu and Brown, 2006). Es werden aber dringend weitere Untersuchungen benötigt, um (a) die biologischen Hintergründe für die postulierten genetischen Effekte zu verstehen und (b) auszuschließen, dass es sich hier lediglich um „Koinzidenzen“ handelt. Die Tatsache, dass wirtsabhängige Faktoren die Varroareproduktion auf individueller Ebene beeinflussen und dass es hierfür offensichtlich eine genetische Grundlage gibt, ist zunächst ermutigend für weitere Selektionsbemühungen hin zu varroatoleranten Honigbienen.

Die Versuche zur Populationsentwicklung auf Bienenvolkebene zeigen allerdings, dass unter praktischen Bedingungen nicht nur die Wirt-Parasit-Wechselwirkungen auf individueller Ebene eine Rolle spielen. Vielmehr hängt der saisonale Anstieg der Varroapopulation stark von Anzahl und Befallsgrad der anderen Bienenvölker im Flugradius ab. Das Problem der „Varroainvasion“ konnten wir erstmals unter klar definierten Bedingungen quantifizieren. Wir machten uns hierfür den (inzwischen ehemaligen) Truppenübungsplatz in Münsingen auf der Schwäbischen Alb zu Nutzen (Frey et al., 2011). Durch die isolierten Bedingungen auf diesem Militärgelände (keine anderen Bienenstände im Flugradius) konnten wir erstmals die Verbreitung von Varroamilben zwischen Bienenvölkern ohne Beeinflussung durch andere Bienenvölker und Imker aus der näheren Umgebung untersuchen. Wildlebende Honigbienenvölker, die als Schwärme in das 7000 ha große Gelände eingeflogen sein könnten, wurden von uns und den Verwaltern des ehemaligen Truppenübungsplatzes nicht beobachtet. Während des Versuches im Spätsommer variierte der Milbeneintrag in die mit Akariziden dauerbehandelten Versuchsvölker zwischen 85 und 444 Milben pro Volk. Diese Zahl erscheint sehr hoch, da sich außer den von uns aufgestellten vier „Milbenspendervölkern“ keine weiteren Honigbienen auf dem Gelände befanden. Erstaunlicherweise hing der Umfang der eingetragenen Milben nicht von der Entfernung zwischen den Bienenvölkern ab. Selbst eine Distanz von 1,5 km bot keinen Schutz vor Milbeneintrag. Der wöchentliche Milbeneintrag war korreliert mit der Tagestemperatur und damit der durchschnittlichen Dauer an potentiellen Flugstunden der Bienen. Dieses Ergebnis bestätigt, dass die Invasionsrate durch die Aktivität (vermutlich Räuberei) der Flugbienen begünstigt wird (Ritter and Leclercq, 1987; Sakofski et al., 1990; Goodwin et al. 2006). Diese Räuberei würde in Phasen mit geringem Nahrungsangebot für die Bienenvölker vermutlich noch ansteigen (Greatti et al., 1992) und somit dürfte dann auch die Invasionsrate zunehmen.

Mit diesem Versuchsansatz konnte zwar sehr genau die Invasionsrate in Abhängigkeit von Entfernung und Befallsgrad definierter „Milbenspendervölker“ quantifiziert werden. Es war aber keine Aussage darüber möglich, welchen Effekt dieser Milbeneintrag hätte, wenn sich die eingetragenen Milben noch zusätzlich reproduziert

hätten. Nach Fries et al. (1994; 2003) kann sich der Varroabefall bei normaler Reproduktion monatlich verdoppeln. Dieser Aspekt konnte im ersten Versuchsansatz nicht erfasst werden, da alle Versuchsvölker mit Akariziden dauerbehandelt wurden und somit alle eingetragenen Varroamilben sofort abgetötet wurden. Zudem hatten wir in diesem Versuchsansatz die Milbeninvasion nur über eine relativ kurze Zeitspanne erfasst und bei einer vergleichsweise geringen Bienendichte. Diese ist in vielen Regionen mit bis zu 4,35 Völkern pro km^2 sehr viel höher als auf dem isolierten Gelände des Truppenübungsplatzes. In einem weiteren Experiment wurde daher das Versuchsdesign verändert und stärker an den praktisch-imkerlichen Bedingungen ausgerichtet (Frey and Rosenkranz, 2014). Der Umfang und zeitliche Verlauf der Milbeninvasion im Spätsommer wurde an zwei Standorten untersucht, die sich durch die Anzahl der Bienenvölker im Flugradius unserer Versuchsvölker unterschieden (geringe und hohe Bienendichte). An beiden Standorten wurde aber nicht nur die Milbeninvasion, sondern auch in einer weiteren Versuchsgruppe das Vermehrungspotential der von außen eingetragenen Varroaweibchen erfasst. Damit wurde eine Situation simuliert, wie sie in der imkerlichen Praxis häufig vorkommt: Bienenvölker werden im Juli gegen die Varroose behandelt und bis zur Winterbehandlung im November nicht mehr auf Varroabefall kontrolliert. In diesem Versuch wurde nun der Anstieg der Varroapopulation durch Eintrag und anschließende Vermehrung dieser Milben überprüft. Zusätzlich wurde die Populationsdynamik der Bienenvölker (n gesamt = 28) von Juli bis zum darauffolgenden Frühjahr regelmäßig nach der Liebefelder Schätzmethode erfasst (Imdorf et al., 1987).

Zwischen beiden Standorten, aber auch zwischen den einzelnen Völkern, variierte die Anzahl der eingetragenen Milben stark. Aufgrund der guten Pollen- und Nektarversorgung an beiden Versuchsstandorten kann dies hier jedoch nicht auf ein mangelndes Nahrungsangebot für die Bienenvölker und eine damit verbundene, verstärkt auftretende Räuberei zurückgeführt werden. Am Standort mit hoher Bienendichte wurden zwischen 266 und 1171 Milben pro Volk über den gesamten Versuch hinweg eingetragen, was einem Durchschnitt von > 450 Milben pro Volk entspricht. In die Bienenvölker am Standort mit einer geringen Bienendichte in der

Umgebung wurden dagegen insgesamt nur ca. 125 Milben pro Volk von außen eingetragen. Diese unterschiedlichen Invasionsraten sind eindeutig durch die hohe Bienendichte am ersten Standort zu erklären, an dem sich über 300 Bienenvölker mit unbekanntem Varroabefallsgrad im Flugradius unserer Versuchsvölker befanden. Die Invasionsraten sind insgesamt geringer als die zu Beginn der Varroaverbreitung von Greatti et al. (1992) angegebenen Zahlen. Dies dürfte daran liegen, dass an unseren Versuchsstandorten (a) es wohl keine wildlebenden Schwärme gab und (b) die meisten Imker inzwischen eine Varroabekämpfung durchführen und damit extreme Befallszahlen vermeiden.

Entsprechend des hohen Invasionsdruckes am Standort mit hoher Bienendichte stieg die Varroapopulation um mehr als das 25-fache in einem Zeitraum von nur drei Monaten an. Am Standort mit geringer Bienendichte nahm die Milbenpopulation im selben Zeitraum dagegen nur um das 3,5-fache zu. In einem langjährigen bundesweiten Monitoringprojekt, das die periodischen, hohen Verluste von Bienenvölkern während des Winters untersucht, wurde bestätigt, dass ein Befallsgrad der Adultbienen von ca. 6% im Spätherbst - kurz vor der Einwinterung - ausreicht, um die Überwinterung der Völker zu gefährden (Genersch et al., 2010). Dieser Schwellenwert, der auch von anderen Autoren bestätigt wird (Delaplane and Hood 1999, Strange and Sheppard 2001, Currie and Gatien 2006), wurde in den sieben Völkern am Standort mit hoher Bienendichte bereits Ende September mit durchschnittlich 6,5 % Bienenbefall erreicht, während die Völker am Standort mit geringer Bienendichte unterhalb der Schadensschwelle blieben. Besonders interessant war, dass die stark parasitierten Völker trotz der Entfernung nahezu aller Milben kurz vor der Einwinterung über 60% ihrer Bienen während der Überwinterung verloren. Dieser Bienenverlust war signifikant höher als bei den schwächer befallenen Versuchsvölkern. Die gute Volksentwicklung aller vier Versuchsgruppen während des Spätsommers sowie der überdurchschnittlich hohe Verlust an Bienenmasse während des Winters sind noch einmal in Tabelle I dargestellt (nicht in der Veröffentlichung enthalten).

Tab. I: Durchschnittliche Anzahl Bienen und Brutzellen der Versuchsvölker des zweiten Versuches zur Reinvasion (Frey and Rosenkranz, 2014). Die unbehandelte Versuchsgruppe am HBD-Bienenstand hatte den höchsten Varroabefall im Oktober und verlor danach überdurchschnittlich viele Bienen während der Überwinterung bis zum Februar.

Datum		**GBD**		**HBD**	
		behandelte Völker (n = 7)	unbehandelte Völker (n = 7)	behandelte Völker (n = 7)	unbehandelte Völker (n = 7)
26. Juli	Bienen	18.585 ± 4.120	17.232 ± 6.050	21.636 ± 7.142	20.866 ± 6.175
	Brut	25.971 ± 4.742	19.000 ± 8.270	30.686 ± 6.292	31.486 ± 10.313
16. August	Bienen	-	17.488 ± 3.071	-	25.350 ± 2.993
	Brut		27.233 ± 7.734		34.114 ± 5.306
05. September	Bienen	17.510 ± 3.645	14.486 ± 4.205	18.359 ± 3.718	22.631 ± 3.506
	Brut	17.857 ± 4.787	14.886 ± 4.740	15.143 ± 2.622	22.400 ± 2.982
26. September	Bienen	-	13.464 ± 2.614	-	17.696 ± 5.612
	Brut		12.914 ± 3.951		16.257 ± 2.973
17. Oktober	Bienen	12.639 ± 3823	9.791 ± 1.906	10.266 ± 2.249	14.176 ± 1.763
	Brut	3.400 ± 1.553	857 ± 964	3.400 ± 2.179[1]	4.971 ± 2.570
28. Februar	Bienen	7.614 ± 4.103	6.268 ± 9.80	7.800 ± 1.892	5.934 ± 1.382
	Brut	2.800 ± 2.361	2.314 ± 1.904	3.686 ± 1.747	2.943 ± 2.190

Von unserem Kooperationspartner wurden die Bienen der verschiedenen Versuchsgruppen vor und nach dem Winter auf Befall mit Bienenviren untersucht. Bei den stark mit Varroa parasitierten Völkern waren vor dem Winter deutlich höhere Virenbelastungen diagnostiziert als in den schwächer befallenen Völkern. Im Frühjahr war allerdings nahezu kein Virusbefall mehr feststellbar (McMahon et al., in Vorbereitung). Die wahrscheinlichste Erklärung dafür ist, dass die mit Viren belasteten Winterbienen nicht wie üblich vier bis sechs Monate lebten (Mattila et al., 2001), sondern deutlich früher abgingen. Dies bestätigt zum einen die Ergebnisse von Amdam

et al. (2004) und Van Dooremalen et al. (2012), die für Varroa parasitierte Winterbienen eine kürzere Lebensdauer nachwiesen, und unterstützt des Weiteren Vermutungen, dass die Varroaschäden, insbesondere durch Varroa bedingte Winterverluste (Genersch et al., 2010; Guzmán-Novoa et al., 2010), maßgeblich durch Virenbefall ausgelöst werden (Nguyen et al. 2011, Francis et al. 2013).

Mit den Ergebnissen zur Milbeninvasion, der Varroapopulationsdynamik im Spätsommer und Herbst sowie dem Einfluss des Varroabefalls auf die Überwinterung von Bienenvölkern lassen sich zunächst klare Empfehlungen für die Imkerpraxis ableiten: (1) Die Imker sollten alle Bienenvölker einer Region möglichst zur gleichen Zeit behandeln, um den Invasionsdruck durch nicht behandelte Bienenvölker („Varroaspender“) zu vermeiden. (2) Der Imker darf sich auch nach einer korrekt durchgeführten Varroabehandlung im Spätsommer nicht sicher sein, dass die Völker gesund eingewintert werden; regelmäßige Befallsdiagnosen sind unbedingt notwendig. (3) Die Aufstellung sehr vieler Bienenvölker in einer Region sollten wenn möglich vermieden werden.

Darüber hinaus weisen diese Ergebnisse aber auch auf generelle Probleme bei der Etablierung eines stabilen Parasit-Wirt-Verhältnisses hin, unabhängig davon, ob dies durch natürliche Selektion (Fries et al, 2006) oder gezielte Zuchtauslese (Büchler at al., 2010) erreicht werden soll. Der Superorganismus Honigbienenvolk vermehrt sich über die Teilung des Volkes (Schwärmen), was von großer Bedeutung für die Fitness des Bienenvolkes ist. Die Übertragung von Pathogenen kann entweder horizontal (zwischen Individuen innerhalb einer Generation) oder vertikal (zwischen Individuen von einer Generation zur nächsten) vonstattengehen. Im Bienenvolk laufen diese Übertragungswege zusätzlich noch auf zwei Ebenen ab, innerhalb des Bienenvolkes und zwischen benachbarten Bienenvölkern (Fries and Camazine, 2001). Über das Schwärmen werden Pathogene, darunter auch die Varroamilbe, vertikal übertragen. Der horizontale Übertragungsweg tritt innerhalb des Volkes durch den Wechsel von Biene zu Biene oder von Biene zu Larve und zwischen den Völkern durch Verflug infizierter Bienen oder Ausräubern befallener Völker auf. Im Bezug auf die Varroamilbe als neuer Parasit unserer Bienenvölker ist vor allem die horizontale Übertragung von Bedeutung.

Man geht davon aus, dass Pathogene, die vorwiegend an den horizontalen Übertragungsweg gebunden sind, eine höhere Virulenz gegenüber ihres Wirtes aufweisen und Pathogene mit überwiegend vertikaler Übertragung sich eher in Richtung stabiler Wirt-Parasit-Systeme entwickeln (Fries and Camazine, 2001). Die hier durchgeführten Versuche zeigen eindrucksvoll, dass die Imkerpraxis die horizontale Verbreitung von Varroamilben fördert. Virulente Milben, die ihr Wirtsbienenvolk umbringen, haben durch diesen Verbreitungsweg eine große Chance, benachbarte Bienenvölker zu befallen. Varroamilben mit moderater Virulenz haben unter diesen Bedingungen keinen Selektionsvorteil. Zudem wird durch die regelmäßige Bekämpfung der Varroamilbe, die in Deutschland aus seuchenrechtlichen Gründen verpflichtend ist, verhindert, dass anfällige Bienenvölker aus der Population ausselektiert werden.

Damit befindet sich die Bienenwissenschaft und Zuchtpraxis in einem Dilemma: Eine Reduzierung der Bekämpfung dürfte zwar im Sinne einer natürlichen Selektion dazu führen, dass anfällige Bienenvölker aus der Population verschwinden. Solange die Imker aber für eine hohe Bienendichte und damit eine horizontale Verbreitung sorgen, werden virulente Varroamilben weiterhin einen Selektionsvorteil besitzen.

Bezeichnenderweise werden langfristig stabile Wirt-Parasit-Beziehungen in gemäßigten Klimazonen bisher lediglich in nicht imkerlich bewirtschafteten Honigbienen-populationen bei relativ geringer Bienendichte beobachtet (Le Conte et al., 2007, Seeley, 2007, Locke and Fries, 2011). Diese Populationen zeigen aber auch, dass stabile Wirt-Parasit-Systeme grundsätzlich möglich sind. Meine Ergebnisse zeigen, dass auf individueller Ebene die Steuerung der Varroafertilität einen potentiellen Resistenzparameter des Wirtes mit einer genetischen Basis darstellt. Dies ist vielversprechend und sollte intensiv auf physiologischer und genetischer Ebene weiter bearbeitet werden. Für Selektionsprogramme, insbesondere mit Beteiligung von Imkern, müssen die hier aufgezeigten Wechselwirkungen zwischen den Bienenvölkern einer lokalen Population stärker als bisher berücksichtigt werden.

4 Zusammenfassung

4.1 Zusammenfasssung

Das Honigbienenvolk ist konfrontiert mit einer Vielzahl an Pathogenen, darunter Viren, Bakterien, Pilze und Milben. Der weltweit bedeutendste Parasit der westlichen Honigbiene ist dabei die ektoparasitische Bienenmilbe *Varroa destructor*. Entdeckt wurde die Milbe zu Beginn des 20. Jahrhunderts in Südostasien in Völkern der östlichen Honigbiene *Apis cerana*, ihrem ursprünglichen Wirt. Ab Mitte des letzten Jahrhunderts wurde die Milbe dann durch Transporte infizierter *A. mellifera* Völker weltweit verbreitet mit meist dramatischen Folgen für wildlebende und bewirtschaftete Honigbienenvölker. Mittlerweile stellt der Parasit das größte wirtschaftliche Problem für die globale Imkerei dar. So gibt es in den gemäßigten Breiten nahezu kein varroafreies Volk mehr und ohne regelmäßige Bekämpfung der Varroose durch den Imker gehen Bienenvölker innerhalb weniger Jahre ein. Dies bestätigt, dass sich bei *A. mellifera* nach wie vor kein stabiles Wirt-Parasit-Verhältnis entwickelt hat und die Bekämpfung von *V. destructor* nach wie vor die größte Herausforderung für die Imkerei darstellt.

Die Hauptursache für die Wirtsschädigungen ist die enorme Zunahme der Varroapopulation im Jahresverlauf, die offensichtlich durch das Bienenvolk nicht ausreichend kontrolliert werden kann. Dieses Populationswachstum wird beeinflusst durch Reproduktionsrate der Varroaweibchen innerhalb der einzelnen Brutzellen, durch Parasit-Wirt-Wechselwirkungen auf der Ebene des Bienenvolkes und durch Wechselwirkungen zwischen den Bienenvölkern.

Im Rahmen der vorliegenden Dissertation wurden Untersuchungen auf allen drei Ebenen durchgeführt. Zunächst konnte auf der individuellen Ebene erstmals nachgewiesen werden, dass Signale der Bienenlarve nicht nur die Reproduktion der Varroaweibchen aktivieren, sondern eine begonnene Oogenese sogar wieder stoppen können (Frey et al., 2013). Unter evolutiven Gesichtspunkten macht dies Sinn, da das Varroaweibchen nur dann Ressourcen in die Eibildung investiert, wenn die Bedingungen für eine erfolgreiche Reproduktion gegeben sind. Meine Ergebnisse lassen

vermuten, dass kutikuläre altersabhängige Duftstoffe der Bienenlarve an der Steuerung der Varroareproduktion maßgeblich beteiligt sind. Als Wirtssignale kommen dabei bestimmte Fettsäureester in der polaren Fraktion des Kutikulaextraktes in Frage.

Im Rahmen eines Kooperationsprojektes konnten wir darüber hinaus zeigen, dass die temporär auftretende Infertilität von Varroaweibchen signifikant mit drei QTL auf drei Chromosomen der Wirtslarve korreliert ist, es also offensichtlich eine genetische Basis für solche reproduktionshemmenden Resistenzfaktoren gibt (Behrens et al., 2012). Hierfür machten wir uns die haplo-diploide Geschlechtsbestimmung bei Honigbienen zunutze, da potentielle Resistenzgene eher im haploiden Phänotyp sichtbar werden und sich das Genom leichter molekulargenetisch charakterisieren lässt.

Für Untersuchungen von Varroaresistenz auf der Ebene des Bienenvolkes sowie für die Durchführung von Selektionsprogrammen ist ein weiterer Aspekt von enormer Bedeutung. In zwei Versuchsansätzen konnte ich nachweisen, dass der Eintrag von Varroamilben von benachbarten Völkern – oft als „Reinvasion“ bezeichnet - die Populationsentwicklung des Parasiten im Volk nachhaltig beeinflusst. Zunächst wurde unter den Bedingungen eines isolierten Truppenübungsplatzes der Eintrag von Milben in Abhängigkeit vom Invasionsdruck (= Anzahl und Entfernung infizierter Bienenvölker) quantifiziert. Hierfür wurden mit Akariziden dauerbehandelte Völker in unterschiedlichen Abständen zu stark parasitierten Völkern aufgestellt und der Milbeneintrag wöchentlich erfasst (Frey et al, 2011). In einem weiteren Versuch wurde der Ansatz erweitert: Neben der Varroainvasion wurde nun auch die Vermehrung der eingetragenen Milben quantifiziert. Unter praxisnahen Bedingungen konnte so nachgewiesen werden, dass horizontale Verbreitung plus anschließende Vermehrung der eingeschleppten Varroamilben zu einem exponentiellen Anstieg der Varroapopulation führen können, der innerhalb von drei Monaten die Schadensschwelle übersteigt (Frey and Rosenkranz, 2014) und dadurch die Überwinterung der Bienenvölker gefährdet wird. Aus den Versuchsergebnissen werden Empfehlungen für eine flächendeckende und zeitlich koordinierte Varroabekämpfung sowie für die Selektion resistenter Bienenvölker abgeleitet.

Mit meinen Versuchen konnte ich einige Mosaiksteine zum Verständnis der Varroa-Reproduktionssteuerung innerhalb der individuellen Brutzelle und zur Varroa-Populationsdynamik innerhalb des Bienenvolkes hinzufügen. Ein solches Verständnis der Populationsdynamik und der zugrunde liegenden Wirtsfaktoren ist meiner Meinung nach essentiell für die Selektion varroaresistenter Bienenvölker und darüber hinaus wichtig für die Entwicklung von Varroa-Bekämpfungskonzepten.

4.2 Summary

The honey bee colony is faced with a huge number of pathogens, including bee viruses, bacteria, fungi and mites. Among these pathogens, the ectoparasitic mite *Varroa destructor* is considered the most important parasite of the honey bee worldwide. This mite was discovered at the beginning of the last century in South East Asia within colonies of the original host, the Eastern honey bee *Apis cerana*. From the middle of the last century the mite has been spread worldwide by transports of infested *A. mellifera* colonies with dramatic consequences for both, feral and managed honey bee colonies. In the meantime this parasite has become the most serious economic problem for global beekeeping. In temperate climates nearly all honey bee colonies are infested and without yearly Varroa treatments these colonies would collapse within a few years. This confirms that a stable host parasite relationship has not been established yet. Therefore the control of *V. destructor* still represents the main challenge for beekeeping.

The main reason for host damages is the dramatic increase of the Varroa population during the season. Our honey bee colonies are obviously unable to control this population dynamic of the parasite. The increase of the mite population is influenced by the reproductive rate of Varroa females within individual brood cells, by host-parasite-interactions on the colony level and by interactions among honey bee colonies on the population level.

The dissertation at hand presents experimental approaches and results at all three levels. On the individual level we were able to demonstrate that age-dependent signals of the honey bee larvae not only activate the oogenesis of the Varroa females but even trigger the further course of mite reproduction (Frey et al., 2013). Our studies on the activation of the Varroa reproduction revealed that exclusively larvae within 18 h (worker) and 36 h (drones), respectively, after cell capping were able to stimulate the mite's oogenesis. Furthermore, we were able to confirm for the first time the presence of a signal in the host larvae allowing the reproducing mites to adjust their own reproductive cycle to the ontogenetic development of the host. Under certain conditions such host signals can even stop an oogenesis of the female mite that has already been started. From an

adaptive point of view that sort of a stop signal enables the female mite to save resources for a next reproductive cycle if the own egg development is not sufficiently synchronized with the development of the host. My results indicate that age specific volatiles of the larval cuticle are involved in the regulation of mite reproduction. According to preliminary quantitative GC–MS analysis we suggest certain fatty acid ethyl esters as candidate compounds. These host signals – either involved in the activation or in the interruption of the Varroa reproduction – offer possibilities to influence the reproductive success of Varroa females and might therefore be used for biological control in the future.

Within an EU cooperation project we could additionally demonstrate that the so called temporary infertility of Varroa females is significantly correlated with three QTL of the host larvae. This confirms a genetic basis for host resistance factors that inhibit the mite reproduction (Behrens et al., 2012). For this study we made use of the fact that we had access to a honey bee population at the island of Gotland, Sweden that has survived mite infestation without any treatment for more than 10 years. We crossed a queen from this tolerant population with drones from susceptible colonies to rear hybrid queens and produced a mapping population of haploid drones from these hybrids. Because honey bees have a haplodiploid sex determination, the haploid drones provide an extremely simple and highly efficient model system for genetic studies. Subsequently, we mapped three candidate target regions on chromosomes 4, 7, and 9. Although the individual effect of these three QTL was found to be relatively small, the set of all three had significant impact on the suppression of *V. destructor* reproduction by epistasis. The detection of this epistatic interaction was only possible because we used the simple genetic make-up of haploid drones.

For studies on Varroa resistance on the colony level and for selection programs the interactions among the colonies of the local honey bee population have to be considered. In two experimental approaches I was able to prove that the invasion of Varroa mites from neighboring colonies – often called “reinvasion” – significantly influences the population dynamic of the parasite within the colony. First, we quantified the number of mites invading individual colonies in relation to the invasion pressure (=

number and distance of infested colonies). For this approach we made use of an isolated military training area near Münsingen at the Swabian Alb not accessible to other beekeepers (Frey et al, 2011). We established ten “mite receiver colonies” continuously treated against *V. destructor* and placed them at distances of 1m to 1.5 km from four heavily infested “mite donor colonies”. In the donor colonies, we estimated the population of bees, brood, and *V. destructor* at three week intervals. The invasion of mites into the receiver colonies was recorded every 7-12 days. During the measurement period of about two months, between 85 and 444 mites per colony were introduced into the receiver colonies. Surprisingly, there were no significant differences in the invasion rates in relation to the distance between donor and receiver colonies.

The second approach was performed under more realistic field conditions of two experimental apiaries established in regions with high and low bee densities, respectively. Additionally, in this experiment we analyzed the multiplication of the invaded mites. Thereby we confirmed that horizontal transmission plus the reproduction of the invaded Varroa mites can cause an exponential increase of the mite population that may exceed the damage threshold within three months (Frey and Rosenkranz, 2014). We were further able to show that the invasion rates – and therefore the final infestation – differ significantly according to the number of honey bee colonies in the neighborhood of the apiary: At the site with a high bee density, the average invasion rate per colony over the entire three and a half months period was 462 mites per colony compared to only 126 mites per colony at the site with a low bee density. As a consequence, the colonies of the apiary at the high bee density site revealed an average final infestation in November of 2,082 mites per colony compared to 340 mites per colony at the low bee density site. The highly infested colonies lost about three times more bees compared to the lower infested colonies – obviously a result of Virus infections transmitted by Varroa mites (McMahon et al., in preparation).

With my different approaches I was able to add further elements of knowledge for a better understanding of how host factors and ambient conditions influence the Varroa

reproduction within individual brood cells and the population dynamic within a honey bee colony. A better knowledge of these host parasite interactions is essential for the selection of mite resistant colonies and further more important for the development of concepts for an effective Varroa treatment.

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1. Bei der eingereichten Dissertation zum Thema

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Die entsprechenden Strafvorschriften sind in § 156 StGB (falsche Versicherung an Eides Statt) und in § 161 StGB (Fahrlässiger Falscheid, fahrlässige falsche Versicherung an Eides Statt) wiedergegeben.

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Wer vor einer zur Abnahme einer Versicherung an Eides Statt zuständigen Behörde eine solche Versicherung falsch abgibt oder unter Berufung auf eine solche Versicherung falsch aussagt, wird mit Freiheitsstrafe bis zu drei Jahren oder mit Geldstrafe bestraft.

§ 161 StGB: Fahrlässiger Falscheid, fahrlässige falsche Versicherung an Eides Statt:

Abs. 1: Wenn eine der in den §§ 154 und 156 bezeichneten Handlungen aus Fahrlässigkeit begangen worden ist, so tritt Freiheitsstrafe bis zu einem Jahr oder Geldstrafe ein.

Abs. 2: Straflosigkeit tritt ein, wenn der Täter die falsche Angabe rechtzeitig berichtigt. Die Vorschriften des § 158 Absätze 2 und 3 gelten entsprechend.

Ich habe die Belehrung zur Eidesstattlichen Versicherung zur Kenntnis genommen.

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