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Influence of Neem stem-injection on the reproductive activity of *Ips typographus* (Col., Scolytinae)

Diploma thesis

Master study Phytomedizin

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Zusammenfassung

Neemöl wird aus dem Kernextrakt des tropischen Neembaums *Azadirachta indica,* gewonnen und ist für seine insektizide Wirkung bekannt. Der aktive Wirkstoff Azadirachtin baut sich in der Umwelt sehr schnell ab und eignet sich daher besonders für die Anwendung in ökologisch sensiblen Gebieten. In der vorliegenden Arbeit wurde untersucht, inwieweit sich die Stamminjektion von Neem-Extrakt in lebenden Fichten auf die Bereitschaft der Mutterkäfer oder Jungkäfer des Fichtenborkenkäfers, *Ips typographus* zur Anlage von Folgebruten auswirkt. Da sowohl Geschwisterbruten als auch die Brut der ersten Generation in erheblichen Maße zur Vermehrung der Käfer beitragen, wäre eine Verminderung der Reproduktionsfähigkeit der im behandelten Baum brütenden oder sich entwickelnden Käfer ein entscheidendes Kriterium für die erfolgreiche Anwendung des Neemöl-Stammapplikationsverfahrens im Forst.

Lebende Bäume wurden in den Jahren 2008 und 2009 entweder mit einer 5% Lösung „Neem Pro Tree“ (0,05g Azadirachtin/cm Baumdurchmesser, Trifolio-M GmbH) oder reinem Lösungsmittel (blank Lösung ohne aktiven Azadirachtin Wirkstoff) injiziert. Unbehandelte Bäume dienten als Kontrolle. Die Behandlung im Jahr 2008 erfolgte, um die Persistenz des Wirkstoffes nach einem Jahr im Vergleich zu den 2009 behandelten Stämmen zu testen. Nach erfolgreichem Befall der Bäume durch die Mutterkäfer (Parentalgeneration) im Frühjahr 2009 wurde aus vier verschiedenen Baumhöhen (3, 6, 9 und 12 m) der Versuchsvarianten Segmente entnommen. Die aus diesen Segmenten ausfliegenden Mutterkäfer und die Käfer der ersten Generation (F1) wurden abgesammelt und zum Test ihrer Brutbereitschaft an unbehandelte Stämme angesetzt. Ein Teil der ausgeschlüpften Käfer wurde seziert und der Reifegrad ihrer Gonaden bestimmt. Die Brutsysteme in den Segmenten wurden anschließend auf verschiedene Brutparameter (Dichte der Brutsysteme und Muttergänge, Länge der Muttergänge, Anzahl der abgelegten Eier, Eimortalität, Larvenmortalität) untersucht.

Die Brutbereitschaft, Brutleistung und der Entwicklungszustand der Ovarien der Altkäfer sowie die der Jungkäfer aus den 2008 behandelten und 2009 befallenen Stämmen ließen keine eindeutigen Unterschiede verglichen mit Käfern aus Kontrollbäumen erkennen. F1-Weibchen aus diesjährig behandelten Bäumen zeigten zwar eine unterschiedliche Brutbereitschaft, aber die eingebohrten Käfer wiesen ebenfalls keine Unterschiede in der Brutleistung auf. Eine Wirkung von Neem- Extrakten auf die Ovarienentwicklung konnte deshalb auch bei diesen Käfern ausgeschlossen werden.

Die Untersuchung der Ei- und Larvenmortalität sowie der Ausschlüpfrate in den Baumsegmenten zeigte baumindividuelle sowie höhenabhängige Unterschiede. Nur in einem der drei im Frühjahr 2009 mit Neem behandelten Bäume war die Ei- und Larvenmortalität in den oberen Baumsegmenten höher als in den Kontroll- und Blankbäumen. Einen nahezu um 100% verringerten Ausschlupf zeigten jedoch F1-Käfer, die sich in zwei der drei diesjährig behandelten Bäume entwickelt hatten. Eine erhöhte Ei- und Larvenmortalität sowie verringerte Ausschlupfrate trat in den vorjährig behandelten Bäumen nicht auf. Deshalb kann eine Persistenz von Neem- Extrakten im Baum für die Dauer eines Jahres ausgeschlossen werden.

Da es keine Repellentwirkung von Neem-injizierten Bäumen auf die schwärmenden Käfer gibt, ist damit der Schutz eines Einzelbaums vor Befall unmöglich. Das Stamminjektionsverfahren kann auch nicht zur Bereitstellung von stehenden Fangbäumen empfohlen werden, da eine Injektion zwar den Ausschlupf und die Brutanlage der Jungkäfer erheblich vermindert, jedoch die Fähigkeit der Parentalkäfer zur Anlage von Geschwisterbruten nicht beeinflusst sowie kein vollständiges Abtöten der Nachkommen gewährleistet. Zusätzliche Untersuchungen über den Transfer des Wirkstoffes Azadirachtin im Baum wären notwendig um dessen Effekt auf die Brut besser zu verstehen.

Abstract

The seed kernel extract of the tropical Neem tree *Azadirachta indica* is known for its insecticidal properties. The main active ingredient Azadirachtin degrades quickly and, therefore, is especially valuable for ecologically sensitive areas. In this study it was examined to which extent stem-injection with Neem extracts in living Norway spruce trees is affecting the establishment of subsequent broods by parental beetles and filial beetles of first generation (F1) of the spruce bark beetles *Ips typographus*. Reducing reproductive activity of the beetle is crucial for the implementation of Neem-oil as a control agent as subsequent sister- and F1-beetle broods bear high risk for population outbreaks of the beetle.

Living trees were injected either with a 5% Neem ProTree-solution (0,05 g Azadirachtin/cm tree girth, Trifolio-M GmbH) or a blank solution (solvent solution without active ingredient Azadirachtin) in 2008 and 2009. Untreated trees were used as controls. Trees that were injected in 2008 were compared to trees injected in 2009 in order to test for persistence of the agent in the following year. After infestation of trees by parental beetles in spring 2009, log segments were cut from four different tree heights (3, 6, 9 and 12 m) of each tested tree. Parental and F1-beetles emerging from these segments were either mounted on untreated logs to test their willingness to breed or dissected to measure the maturity stage of their ovaries. The breeding systems in all log segments were analysed on various breeding parameters (density of breeding systems and mother galleries, mother gallery length, number of oviposited eggs, egg mortality, larval mortality).

Parental and F1-beetles from the 2008 treated trees showed no significant differences in willingness to breed, breeding performance and gonadal development compared to controls. F1-females from the recently treated trees revealed lower willingness to breed, however, those who successfully established breeding systems did also not differ in breeding performance. Therefore, similar to adult beetles the gonadal development of F1-beetles was not suppressed.

Egg- and larval mortality as well as number of emerged beetles varied between trees and tree heights of the same tree. Only in one of three trees treated with Neem in spring 2009, egg- and larval mortality was significantly higher in the upper tree heights than in the controls. However, in two of three trees almost 100% reduced emergence of F1-beetles was observed. None of the trees treated the year before showed increased egg- larval mortality or reduced emergence of F1-beetles. Therefore, Neem did not persist longer than one year in the tree.

As Neem stem- injected trees are not repelling attacking beetles, protection of single trees by this method is not possible. Neem controlled a significant amount of the emergence of F1 beetles but the number of adult beetles and its first sister brood remained nearly unaffected. Therefore, the stem-injection is not recommended for the use in living trap trees. Additional studies on the transport mechanisms of Azadirachtin within the tree are recommended to better understand its effects on the brood.

Table of contents

1. Introduction

Norway spruce, *Picea abies* is affected by several biotic factors in Europe of which the bark beetle *Ips typographus* (Col., Scolytinae) is currently of most concern for forestry. Bark beetle outbreaks occur especially after events of wind-throw, snow damage or drought and can cause high tree mortality (Wermelinger et al., 2007). The risk of outbreaks is highest at sites with temperatures sufficient to allow development of two or more bark beetle generations per year (Altenkirch et al., 2002; Baier et al., 2007). Infested trees are killed and timber value is reduced by associated blue stain fungi (Altenkirch et al., 2002). On the other hand, *I. typographus* plays an important role in the ecosystem by accelerating the decomposition of dying trees and making them suitable for other wood decaying fungi and insects (Wermelinger et al., 2007).

Beetles overwinter in the bark of infested trees or in the litter and begin to swarm at air temperatures higher than 16.5°C (Lobinger, 1994) and photoperiods of more than 15 hours light (Dobart, 2006; Baier et al., 2007). Male beetles initiate the attack by boring into the bark to establish nuptial chambers. Aggregation pheromones are released to attract more beetles which helps to overcome resin flow, a defence mechanisms of the tree (Baier, 1996; Byers, 2004). Females attracted by pheromones enter the nuptial chamber and establish mother galleries after mating, in which they lay up to 80 eggs (Wermelinger, 2004). After one to two weeks, larvae hatch from eggs and start feeding for up to six weeks in larval galleries rectangular to mother galleries; at the end of which they pupate in pupal niches. After one to two weeks, filial beetles appear which again need another two to three weeks for their maturation in the bark. During this time, the soft chitinous cuticle hardens and changes colour from light yellow to dark brown. Parental beetles often leave the tree to establish a second brood called sister brood two to three weeks after successful initiation of the first brood. (Schwerdtfeger, 1981; Wermelinger et al., 2007). Depending on bark temperature, *I. typographus* needs about seven to twelve weeks total development time. The thermal sum required to complete development is 557±39 degree days (between lower (8,3°C) and upper developmental threshold (38.9°C) of beetle development) (Wermelinger and Seifert, 1998; Baier et al., 2007). The possibility of completing up to three generations per year along with additional sister broods can contribute to high population growth at low elevation and in warm summers (Postner, 1974; Baier et al., 2007).

In order to control bark beetle populations, priority has to be given to preventive measures. Spruce should not be cultivated extensively outside its natural range or below 600 m a.s.l. (Krehan et al., 2006; Baier et al., 2007). Trees of different ages, inter mixture with other tree species, and tiered forest edges are important measures to prevent wind-throw and consequent outbreaks of bark beetles (Wermelinger et al., 2007).

In order to reduce the risk of infestation of living trees, conventional trap trees are used and have to be deployed at a safe distance from the nearest susceptible tree (Postner, 1974; Nierhaus-Wunderwald and Forster, 2004). Trap trees are more effective to attract large number of beetles than pheromone traps, which proved to catch only about 10% of the existing beetle population (Wermelinger, 2004). However, pheromone traps are important to monitor the flight activity of the beetles. Infestation of trees is confirmed by boring dust accumulating on the bark of trees.

The bark of infested trees has to be removed before parental beetles re-emerge and establish sister broods (two to four weeks or 278 degree days after the beginning of infestation) to effectively reduce the population (Baier et al., 2007). Several *I. typographus* outbreaks following storm events in Europe have shown that beetle population density can quickly build up, if fallen trees are not removed in a timely fashion or treated to inhibit development of broods. (Wermelinger, 2004). Damaged or killed spruce trees may still be suitable for *I. typographus* as breeding substrate (Postner, 1974; Wermelinger et al., 2007). If trap trees or damaged trees cannot be debarked or removed from the forest, topical treatment of bark with insecticides is necessary to prevent emergence of beetles (Schwerdtfeger, 1981; Krehan et al., 2006; Baier et al., 2007).

Currently, pyrethroids are the only registered insecticides for bark beetle control in Austria (BFW, 2011). However, pyrethroids are toxic to water organisms and harmful to other insects such as natural enemies of bark beetles (Schröter and Weigersdorfer, 2007). The injection of systemic insecticides into living trap trees (Naumann et al., 1994; Duthie-Holt et al., 1999; Naumann and Rankin, 1999; Helson et al., 2001; Kolev, 2011) could be one alternative to the use of pyrethroids by topical application on felled trap trees. Injected trees may be used as bait for longer time than the felled traps since they might be more attractive as breeding substrate for *I. typographus* alive. Furthermore, if development and reproduction of beetles in bark of stem-injected trees is inhibited by the systemic compound, death trees don´t have to be removed in time for sanitation. This would offer more flexibility for the forest enterprise on the time when trees should be removed. In theory, systemic compounds are taken up by the tree and distributed in xylem and phloem vessels throughout the tree (Altenkirch et al., 2002).

When successful, the injected insecticide compounds reach the phloem tissue and inhibit the reproduction of beetles. If the systemic compounds injected into the trees prove to have repellent or anti-feeding effects against insects attempting to establish breeding systems, the systemic insecticides might also be considered for the protection of single valuable trees.

The Neem tree is native to the Indian sub-continent but has also been introduced to China, Indonesia, Africa, Central and South America (Morgan, 2009). It is an evergreen broad leaf tree that can grow to a height of 15 to 30m and tolerates drought very well. In the tropics, different parts of the trees are used for heating, construction, medical purposes, or as fertilizers and forage for cattle (Schmutterer, 2005). Besides these uses, extracts of Neem leaves and especially Neem seeds are known to have insecticidal and anti-feeding effects against more than 500 insect pest species around the world (Koul, 2004). The seed extract of the Neem tree *Azadirachta indica* with known systemic properties is biodegradable and non-toxic to water organisms or vertebrates (Niemann and Hilbig, 2000; Morgan, 2009). Furthermore, Neem compounds degrade quickly in the environment due to UV-light radiation and temperature (Dureja and Johnson, 2000; Caboni et al., 2006).

Azadirachtin-A (C_{35} H_{44} O_{16}) is the main active ingredient of Neem seeds. Its chemical structure was first described by Kraus (1985, in Schmutterer, 2005). Other derivatives of Azadirachtin and compounds with similar structure (like Azadirachtin -B, Salannin or Nimbin) are also present in seed extracts and contribute to their insecticidal effects but to a much lower extent. Azadirachtin is found in highest concentration in Neem oil which is extracted from Neem seeds by pressing or through extraction with alcohol or water (Schmutterer, 2005).

Neem is known to have many effects on insects including anti-feeding, growth inhibition, reduced fertility and the disruption of cell division processes. However, the exact mechanisms are still not completely understood. Azadirachtin is known to block the prothoracicotropic hormones PTTH (elicitor to produce the moulting hormone ecdysone) and allatostatins (elicitors to produce the juvenile hormones) responsible for growth and development of larvae and yolk deposition into the eggs of adults (Mordue and Nisbet, 2000; Koul, 2004). At a cellular level, Azadirachtin is thought to prevent the transcription of proteins and the cell division. This directly results in delayed or abnormal moults, increased mortality of larvae or reduced ovary and testes development as has been observed in some insects (Mordue and Nisbet, 2000; Morgan, 2009). Neem seed extracts have already been found to effectively reduce *I. typographus* progeny development (Kreutz, 2007; Kolev, 2011; Weber, 2011) and development of other phloem feeding insects (Naumann et al., 1994; Duthie-Holt et al., 1999; Naumann and Rankin, 1999; Poland et al., 2006; Sibul et al., 2009; McKenzie et al., 2010).

Kolev (2011) examined the effects of "Neem pro tree-5" (Trifolio-M Gmbh, Lahnau, Germany) on progeny development and reproductive activity of *I. typographus* following tree injection (0.05g Azadirachtin/cm BHD) or topical spray application prior to infestation by beetles. One month after stem-injection, egg- larval mortality was generally higher in the Neem-treated trees than in the control trees. However, effects varied between individual test trees and tree heights. Furthermore, emergence of beetles was reduced by about 50% four months after Neem stem-injection. Breeding systems in topically treated logs showed significantly reduced number of larval galleries when applied in spring (Kolev, 2011). However, oviposition of parental beetles in Neem-treated trees was not reduced compared to control. Weber (2011) found that mortality during juvenile development reached up to 100% in logs treated topically before and after infestation by *I. typographus*, but similar to Kolev (2010) he did not observe effects on oviposition and gonad maturation of female beetles in his experiments.

In my study, breeding experiments were conducted to further examine the effects of Neem stem-injection on subsequent broods of *I. typographus*. It was tested whether parental and filial beetles (F1), were able to establish sister- and F2-broods, respectively. Since Kolev (2011) reported that Neem injected into trees can persist for up to four month after treatment, I tested the persistence of Neem also after one year of injection by evaluating egg and larval mortality, emergence of filial beetles of first generation (F1) and breeding activity of emerging beetles on untreated stems.

2. Material and Methods

2.1. Origin of tested trees

The study was conducted in the Schneegattern forest district of the Kobernaußerwald forest (owned and managed by the Austrian Federal Forests, ÖBf AG) in Upper Austria (Fig.1). The elevation of the site is 618 m above sea level. The Kobernaußerwald consists of about 15.000 ha mixed stand with approximately 60% Norway spruce, *Picea abies*, 20-25% European beech, *Fagus sylvatica* and 5-20% silver fir, *Abies alba*. The stands are managed as age class forests with clear cuts.

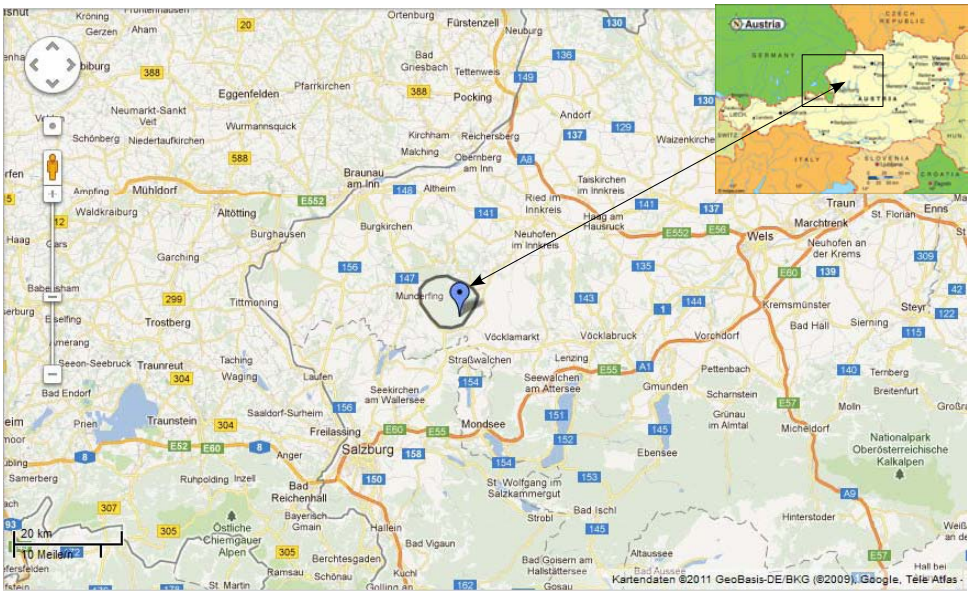


Fig.1: Location of experimental site Kobernaußerwald in Austria.

The climate resembles the mid- European temperate climate. Mean day temperatures range between -2° in winter and 17.1°C in summer. The total precipitation per year is 1229 mm (climate data from weather station of Irrsdorf between 1971 and 2000 (ZAMG, 2011), located at 570m a.s.l., 5km south of the experimental site).

At the study area a total of 17 trees, ranging from 60 to 80 years, were selected, which were located on a south facing exposed slope next to a clearance site where trees had been felled and removed in former years to prevent outbreaks of *Ips typographus* populations. (Fig.2,).

Fig.2: Experimental site in Kobernaußerwald.

2.2. Application of Neem by stem-injection method

The short and long term effects of Neem on reproductive activity of parental beetles, development of filial generation brood (F1) and reproductive activity of F1-beetles were tested by injecting 5% "Neem Pro Tree" solution (Trifolio-M GmbH, Lahnau, Germany; active compound: 0.05 g/ml Azadirachtin) into living trees in 2008 and 2009. Blank injection of solvent solution without Azadirachtin and untreated trees served as controls.

Prior to injection, the trees were inspected for bark beetle infestation. On March 31, 2008, six trees were injected with Neem (further referred to as Neem 08 trees) and two trees were injected with blank solution (further referred to as blank 08 trees). On April 9, 2009, five trees were injected with Neem (further referred to as Neem 09 trees) and again five with blank solution (further referred to as blank 09 trees). Two experimental trees were left untreated as controls. The location of trees at the experimental site subjected to the different treatments are shown in Fig.3.

For injection, 2 cm deep holes, measuring 4 mm in diameter were drilled into the bark around the circumference of the trees at breast height (= 1.3 m). The distance between the drillings was ca. 6.3 cm (~2π). The holes were closed with plastic caps. 2 ml of the Neem or blank solution was applied with the "Wedgle Direct-Inject" Arbor-System (Trifolio-M Gmbh, Lahnau, Germany) through the cap, resulting in a concentration of 0.05g Azadirachtin/cm stem diameter.

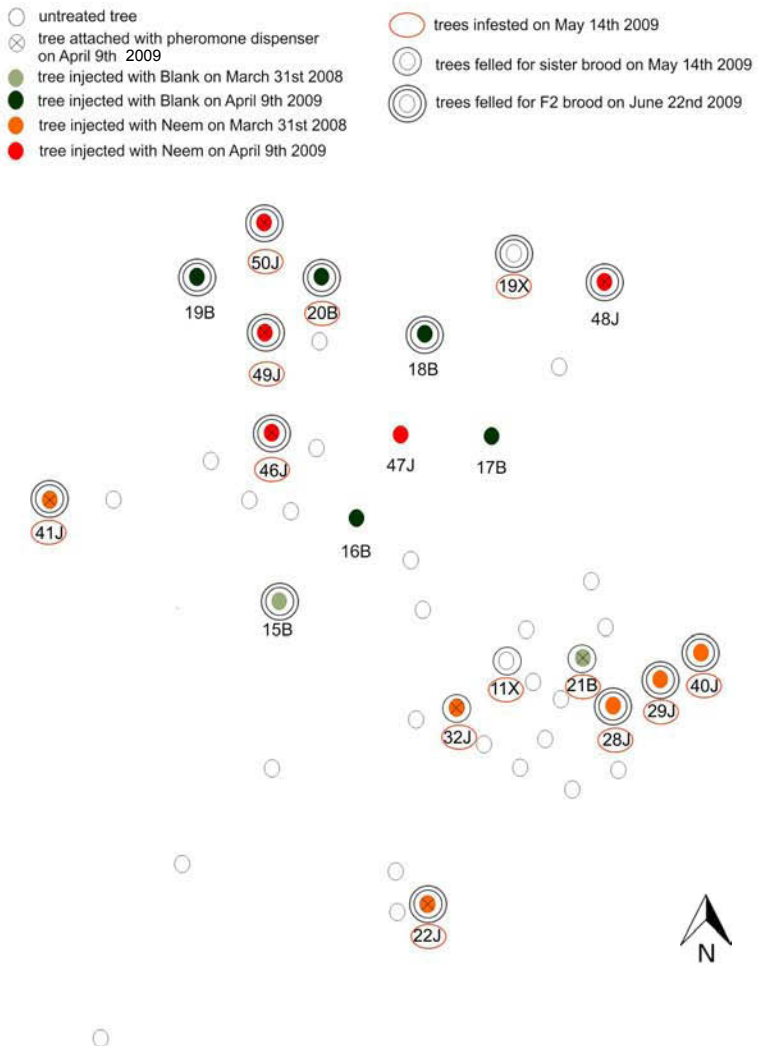


Fig.3: Composition of trees subjected to the various treatments, Neem 2008 injected trees (22J-41J), Neem 2009 injected trees (46J-50J), blank 2008 injected trees (15B, 21B), blank 2009 injected trees (16-20B) and untreated control trees (11X, 19X) at the experimental site in Kobernaußerwald.

2.3. Estimating flight of parental beetles and sampling of trees

To induce infestation of experimental trees (blank or Neem-injected in 2008 and 2009 as well as untreated control) by parental beetles swarming in spring 2009, pheromone dispensers (Ipsowit; Witasek Pflanzenschutz GmbH, Feldkirchen, Austria) were attached to nine of 17 tested trees at a stem height of 2.5 m on April 9, 2009. The trees with attached pheromone dispensers were located at the edge of the forest stand and they were at a maximum distance of 30m from each other to attract beetles evenly to all experimental trees at the study site (Fig.3).

The onset of parental beetle flight activity was ascertained by *I. typographus* pheromone traps operated by the Federal Research Centre for Forests (BFW) at stations in the vicinity of the experimental site (Fig.4). In order to confirm infestation at the experimental site, tested trees were further controlled for the presence of ejected boring dust.

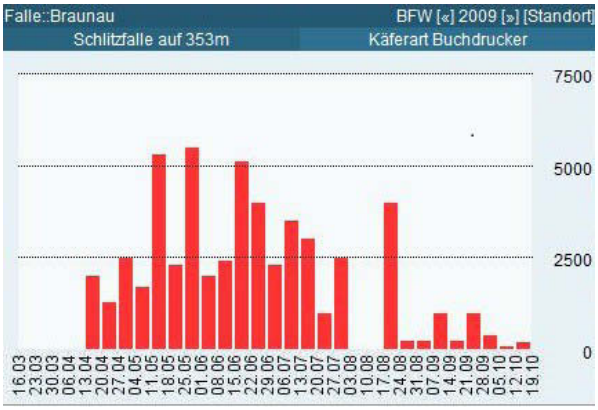


Fig.4: Number of *I. typographus* caught per week in one pheromone trap at Braunau, Upper Austria (353m a.s.l, 30km north-west of the experimental site) during the year 2009 (BFW, 2009).

Test trees of each treatment were either cut on May 14 to collect parental beetles or on June 22 2009 to collect emerging F1-beetles. The felling date was determined using the phenology model PHENIPS (Baier et al., 2007) based on solar radiation and accumulated effective temperature sums of *I. typographus*. Therefore, mean effective day temperatures were added beginning on first day of beetles´ flight activity. Trees were felled when sums of 99 degree days and 225 degree days were reached to catch parental beetles and F1-beetles, respectively. The felling date of each tree and their successful infestation at the experimental site is shown in Fig.3.

From each infested tree, felled either in May or June 2009, 70 cm long log segments were cut from 3, 6, 9 and 12 m tree height (Fig.5). The logs were cut again tangentially to get stem segments with approximately 12 dm^2 (≈ paper format A3) size of bark area. (Fig.6). All log segments were brought to the laboratory to collect the emerging beetles and for further analysis.

Fig.5: Cutting of log segments at 3,6,9 and 12 m tree height

2.4. Collecting of emerging beetles, natural enemies and analysis of breeding systems

In order to collect re-emerging parental beetles, segments from lower tree heights 3 m and 6 m as well as from upper tree heights 9 m and 12 m of each tree were stored together in photo-eclectors in the laboratory at 24°C, 16:8 L:D photoperiod (16 hours of light and eight hours of darkness). The photo-eclectors were constructed from wooden boxes (small boxes: 66 x 31 x 31 cm or large boxes: 71x 45 x 45 cm) with an opening of ca. 20 mm in diameter cut into one end of each box. Beetles emerging from the bark could leave the eclectors only through the opening where they were collected by a small plastic box (Fig.6).

Fig.6: Segment (l) and photo-eclector (r) with plastic box to collect beetles

The number of emerging parental and F1-beetles were counted daily. About 15 female beetles from each tree (depending on availability) were used to assess the maturity of their ovarioles. Further, emerging parental and F1-beetles were used for additional experiments to test their willingness to establish sister broods and F2-broods, respectively. Emerging specimens of natural enemies were also collected and identified after Schmiedeknecht (1930), Hedquist (1963) and Kenis et al. (2004).

One week after emergence of beetles had ceased, the bark of the segments was removed and the breeding systems were analysed for the following parameters:

- Number of breeding systems (BS) per dm² bark area
- Number of mother galleries (MG) per dm² bark area
- Number of beetles in breeding galleries
- Length of mother galleries (MG length)
- Number of egg niches per mother gallery (Eggs/MG)
- Number of egg niches in mother gallery beyond last established larval gallery (Eggs/MG beyond last LG)
- Number of larval galleries per mother gallery (LG/MG)
- Number of incomplete larval galleries (incomplete LG/MG). Larval galleries were considered to be "incomplete" when they were at least 1 cm shorter than the adjacent, younger larval gallery.
- Length of mother gallery beyond last established larval gallery (MG length beyond last LG)

Due to high densities of breeding systems, nine sampling points per segment were selected evenly distributed over the bark area (Fig.7). Next to these sampling points, nine breeding systems (complete and not damaged by maturation feeding) were analysed.

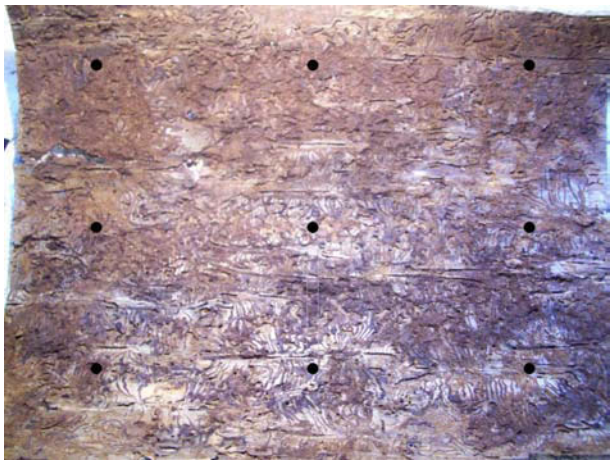


Fig.7: Sampling points (black) on 12 m² large bark piece

Following parameters were calculated from breeding systems:

- Density of breeding systems (BS): $\frac{number\ of\ BS}{dm^2\ bark\ area}$
- Density of mother galleries (MG): $\frac{number\ of\ MG}{dm^2\ bark\ area}$
- Mother gallery length after last established larval gallery (LG) in percent of total length:

$$\frac{MG\ length\ beyond\ last\ LG}{total\ MG\ length} \times 100$$

- Egg density per cm mother gallery: $\frac{eggs\ per\ MG}{MG\ length}$
- Egg mortality in percent of eggs until last larval gallery:

$$\frac{Eggs\ per\ MG\ until\ last\ LG - LG\ per\ MG}{Eggs\ per\ MG\ until\ last\ LG\ (a)} \times 100$$

Since it could not be ascertained whether eggs beyond the last established larval gallery did not hatch yet due to late oviposition or due to mortality, these eggs were excluded from the calculation (Fig.8).

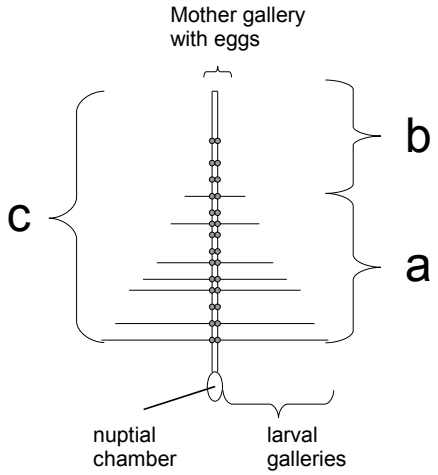


Fig.8: Scheme for calculating of egg-mortality with eggs until last established larval gallery (a), eggs beyond last established larval gallery (b) and total eggs per mother gallery (c).

- Larval mortality in percent: $\frac{LG\ per\ MG - aborted\ LG\ per\ MG}{LG\ per\ MG} \times 100$

2.5. Breeding experiments

The main objective of the study was to find out whether the breeding activity of *I. typographus* was affected by feeding on bark of Neem-injected trees compared to beetles emerging from blank-injected trees or untreated control trees.

For the set-up of the breeding experiments, male and female beetles were differentiated according to morphological criteria: females have a smaller frontal tubercle on the frons and a higher density of bristles on the anterior part of the pronotum than males (Schlyter and Cederholm, 1981).

Male and female beetles were fixed to ~1.2 m long untreated spruce logs by means of the capsular pit method (Führer, 1977). The capsular pits consist of a plastic cup of 20 mm diameter, held in place by an aluminium plate. The aluminium plate is used to fix the plastic cup with an elastic band to the log. A ring of foamed rubber between the bark and the aluminium plate prevents beetles from escaping (Fig.9 a,b). Inside the cup, the beetles had free access to the bark. Male beetles were put into the capsules first. When they had successfully entered the bark on the following day, a female was added to the same capsule. When this female again had entered the bark successfully, another female was added two days later. Beetles that did not bore into the log were replaced the following day. Generally, those beetles were used for the breeding experiments, which had emerged from the segments of the treated trees at the same day. If there were not enough beetles available, they were taken from collected beetles that had emerged two to three days before. The experiments were conducted in outdoor cages (summer conditions) at the garden of the Institute in Vienna, Austria.

Fig.9 a,b: Log with capsular pits (a) and different parts used for the capsular pit experiment (b).

Parental beetles were mounted to uninfested logs felled in November 2008 (Nov 08 logs) and in April 2009 (Apr 09 logs) respectively. F1-beetles were only mounted to breeding logs felled in April 2009. Logs for the breeding experiments with F1-beetles were cut from two uninfested trees. Thus, differences in attractiveness or breeding quality of the log segments had also to be checked.

To assess successful establishment of nuptial chambers and mother galleries, the bark was removed 9-18 days after onset of breeding experiments with F1-beetles and 30-38 days after start of breeding experiments with parental beetles. Similar to the log segments from the field, breeding systems in the experimental logs were analysed for the following parameters: length of mother gallery, number of egg niches, number of larval galleries and number of incomplete larval galleries. These parameters were further used to calculate egg density, egg mortality and larval mortality in sister and F2-broods.

2.6. Examination of gonads

The size of the ovarioles of female parental and F1-beetles which emerged from blank or Neem-treated trees was measured to determine the state of gonad maturation. Ovaries were dissected under a microscope and prepared for photography (Nikon Eclipse, Photo Head V-TP; 20 x magnification) (Fig.10). The area of each of the four ovarioles on the digital image was measured using the program Datinf® Measure 2.1 (Dat Inf GmbH, 2007). To check for already accomplished oviposition, the ovaries of parental females were examined for the presence of the corpus luteum, a yellowish-brown remnant of follicle tissue that remains attached to the base of the ovarioles and oviduct after egg laying.

Fig.10: Ovary of a young *I. typographus* female.

2.7. Statistical analysis and Overview

Statistical analysis was performed using SPSS™ 15 (SPSS Inc, 2006). All percent values were arcsin transformed. Normal distribution of data was confirmed by Kolmogorow-Smirnow Test. Differences between means were tested by Student´s t-test and One Way Anova. Homogeneity of variance was checked by Levenes´ test. Scheffe´s homogeneous post-hoc test and Tamhane non-homogeneous post-hoc test results were computed for testing the level of significance.

Differences of frequencies (established nuptial chambers, mother galleries and feeding galleries as well as boring activity) were tested with the χ^2 analysis, using Fisher´s correction if sample size was below 20 and Yates correction if sample size was between 20 and 200 (Bosch, 1996; Sachs, 2004).

Tab.1 gives a summary about the work events during the study.

Tab.1: Timetable of the study.

Treatment of standing trees	
March 31, 2008	First injection (blank 08 and Neem 08 treatment)
April 9, 2009	Second injection (blank 09 and Neem 09 treatment)
	Attaching of pheromones to trees
April 13-20, 2009	pheromone traps indicate onset of parental beetles swarming

Tested generation: parental beetles and their sister brood	
May 14, 2009	Felling three test trees (one blank 08, one Neem 08 treatment and one control)
May 14-31, 2009	Collection of emerging parental beetles and examination of female ovaries from test trees.
May 21-24, 2009	Breeding experiments with parental beetles (collected from test trees) on untreated logs (logs felled in November 2008)
May 29 – June 4, 2009	Breeding experiments with collected parental beetles (collected from test trees) on untreated logs (logs felled in April 2009)
June 5- 9, 2009	Removing bark of log segments from test trees
June 21- 24, 2009	Removing bark of untreated logs (logs felled in November 2008)
June 29- July 6, 2009	Removing bark of untreated logs (logs felled in April 2009)

Tested generation: F1-beetles and their F2-brood	
June 22, 2009	Felling the rest of the test trees (blank 09, Neem 08, Neem 09 treatment and control)
June 22 - July 16, 2009	Collection of F1-beetles and examination of female ovaries from test trees.
July 2- 10,2009	Breeding of collected F1 beetles on untreated logs
July 12- 19, 2009	Removing bark of test tree log segments
July 17- 19 and July 27, 2009	Removing bark of untreated logs

3. Results

3.1. Reproductive activity and juvenile development in standing trees

The bark of log segments from four tree heights (3, 6, 9 and 12 m) were checked for various breeding parameters (mother gallery length, number of eggs, egg density, egg mortality, larval mortality) to examine effects of Neem treatment on reproductive activity and development of beetles in injected trees.

Since the status of the breeding systems (number of eggs per mother gallery, egg density per cm mother gallery) of the tested log segments did not differ significantly between same treatment of trees felled on May 14 and those on June 22, it could be assumed that parental beetles had already finished egg laying at the first date of felling.

Egg- and larval mortality as well as the emergence of beetles differed among segments within and between trees. Due to this high variability, all breeding parameters are presented separately for each tree height and tree (Fig.12-20).

Intensive maturation feeding of juvenile beetles on one hand or low infestation of the logs by females on the other hand led to low numbers ($n<3$) of breeding systems that could not be analysed statistically.

3.1.1. Emergence of beetles

Neem treatment in 2008 had no obvious effects on re-emergence of parental beetles compared to the control and the blank tree (Fig.11). Therefore, feeding in those trees did not decrease their willingness to re-emerge and infest new trees. Low emergence from lower stem segments of the control tree was due to low infestation by beetles.

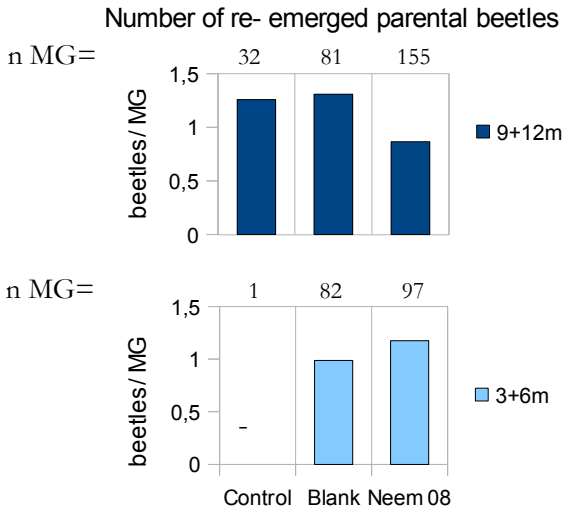


Fig.11: Number of re-emerged parental beetles per mother gallery (MG) from combined log segments of lower (3 m, 6 m) and upper tree heights (9 m, 12 m). Beetles emerged from one tree treated with solvent solution in 2008 (blank), one tree injected with Neem in 2008 (Neem 08) and one untreated control tree. "-": The control tree was barely infested at the lower tree heights therefore no emergence could be recorded from those heights. "nMG": number of mother galleries.

The number of F1-beetles which emerged from the upper stem sections (9+12 m) of Neem 09 treated trees was markedly reduced compared to the control tree. Except for one tree (49J), this was also the case for the lower sections (3+6 m). A lower emergence occurred in two out of five Neem 08 trees (40 J, lower logs of 28J).

Interestingly, less beetles emerged from the blank-injected trees compared to the other treatments, but many beetles were encountered during maturation feeding in the bark of this tree at the end of the experiment (Fig.12).

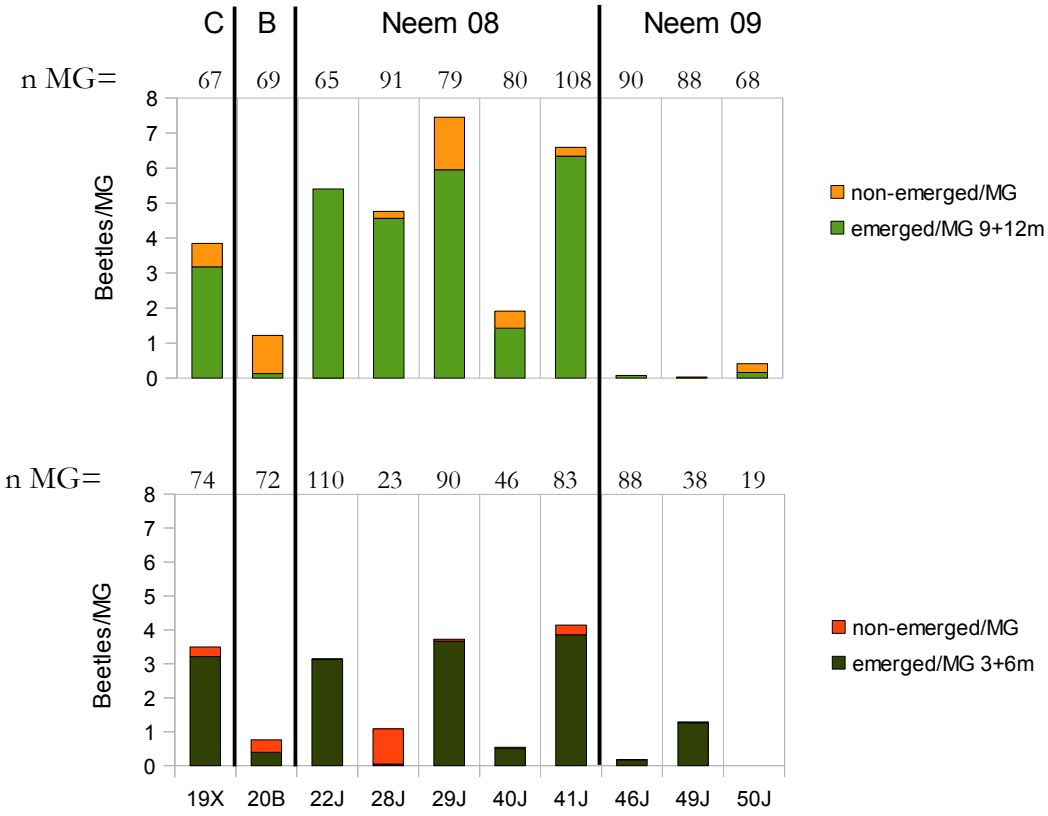


Fig.12: Number of emerged and non-emerged filial 1 beetles (F1) per mother gallery (MG) from combined log segments of lower (3 m, 6 m) and upper tree heights (9 m, 12 m) and different treatments. Tree 19X was untreated (control), tree 20B was injected with solvent solution in 2009 (blank), trees 22J- 41J were injected with Neem in 2008 (Neem 08) and trees 46J-50J were injected with Neem in 2009 (Neem 09). Non-emerged beetles are those that were found in breeding systems after removal of bark. "nMG": number of mother galleries.

There was a difference in the emergence patterns of F1-beetles between the blank tree (20B) and Neem 09 tree 50J on the one hand and control tree and Neem 08 trees on the other hand. A low number of beetles from the blank and Neem 09 tree emerged early after segments were stored in photo-eclectors while emergence of F1-beetles from Neem 08 tree 40J and the control tree started one week later than in the former mentioned trees and was not finished at the end of the experiment (Fig. 13). Beetles from the blank tree emerged earlier than those from Neem 08 trees and most of them were still arrested inside the log at the end of the incubation period.

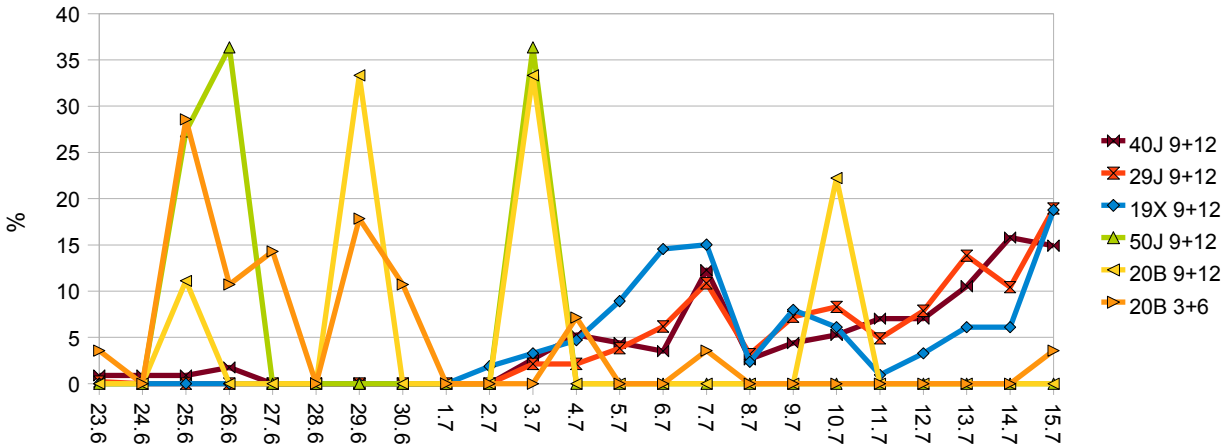


Fig.13: Course of F1-beetles emergence (%) per day of log segments from lower (3 m, 6 m) and upper tree heights (9 m, 12 m). Only log segments are shown with more than 10% of beetles found in the breeding galleries. Lower tree heights of Neem 08 tree 28J and Neem 09 tree 49J are not included as less than four beetles emerged. Trees were injected either with blank solution in 2009 (20B), injected with Neem in 2008 (29J 9+12 m, 40J 9+12 m), injected with Neem in 2009 (50J 9+12 m) or left untreated for control (19X 9+12 m).

In summary, injection with Neem in 2009 generally reduced both, development and emergence of F1-beetles. Neem 08 treatment showed no clear trend compared to the control but for most of the trees emergence was in the same range or higher than from the control tree. Additionally, in blank trees but not in Neem 08 trees emergence from bark seem to be diminished.

3.1.2. Density of breeding systems

The density of nuptial chambers indicates the attractiveness of the various treated trees for male beetle attack (Fig.14 a). On the other hand, the density of mother galleries is showing the total number of parental females per dm^2 bark area attracted by the boring activity of males (Fig.14 b). A low density of both could indicates repellent effects of the treatments.

Neem stem-injection in trees did not reduce the number of beetles attacking the tree. Trees of different treatments, one Neem 09, one Neem 08 and one control tree were not infested at the lower tree heights (Fig.14a,b). Non-infested trees were found for two different treatments (two Neem 09 and five blank trees) (Fig.15). Furthermore, Neem 09 treated trees showed as much or even higher density of breeding systems and mother galleries than control trees (Fig.14a,b). As can also be seen in Fig. 15, pheromone batches on the tree did not influence attack activity.

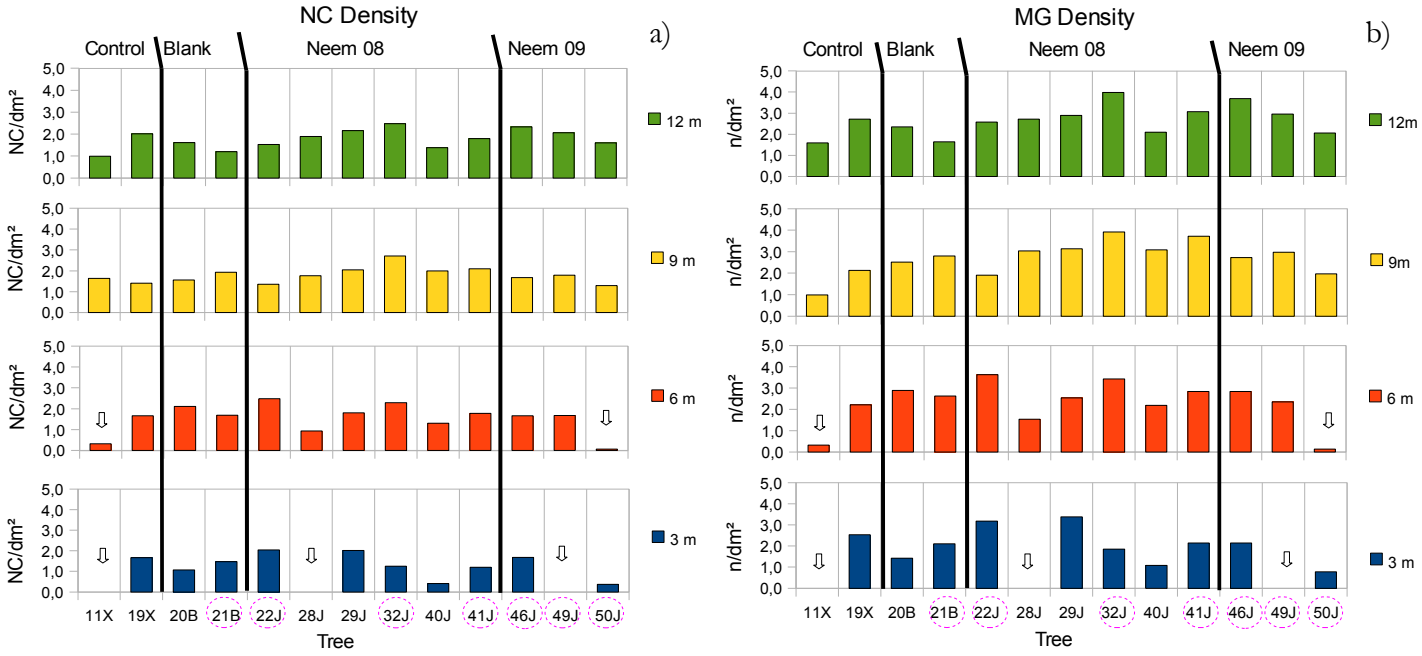


Fig.14 a,b: Density of nuptial chambers (NC)/dm² bark (a) and density of mother galleries (MG)/dm² bark (b) at different tree heights and treatments. Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Log segments with less than three breeding systems (low infestation) are marked with white arrows. Tree numbers marked with pink circle indicate trees with attached pheromone dispensers.

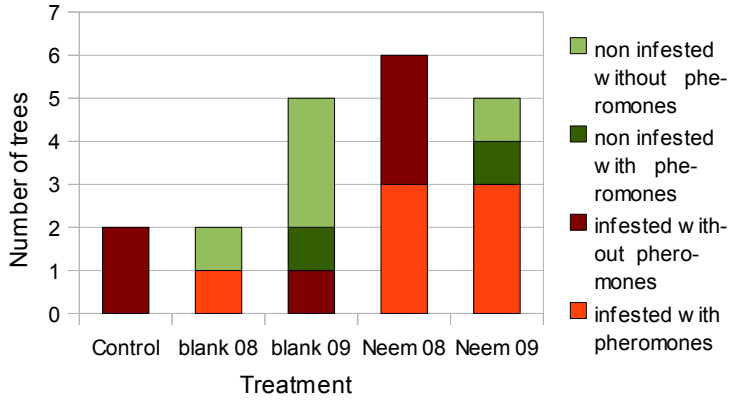


Fig.15: Number of infested and non-infested trees that were attached with pheromone dispensers. Trees were treated with either solvent solution blank in 2008 (blank 08), solvent solution blank in 2009 (blank 09), Neem in 2008 (Neem 08), Neem in 2009 (Neem 09) or left untreated for control.

3.1.3. Length of mother galleries

The length of mother galleries was measured to evaluate whether treatment with Neem reduced feeding or oviposition by female beetles.

Mother gallery length in Neem-treated trees was not reduced compared to control trees. Therefore, Neem stem-injection had no effect on feeding and reproductive activity of parental beetles. Differences in length were only found between upper and lower portions of the control tree (19X) and between different portions of one Neem 08 tree (29J). Mother galleries were longer at lower tree heights in the control tree (19X) than at 12 m. On the other hand, mother galleries were significantly smaller at lowest heights in the Neem 08 tree (29J)($P<0.05$)(Fig.16).

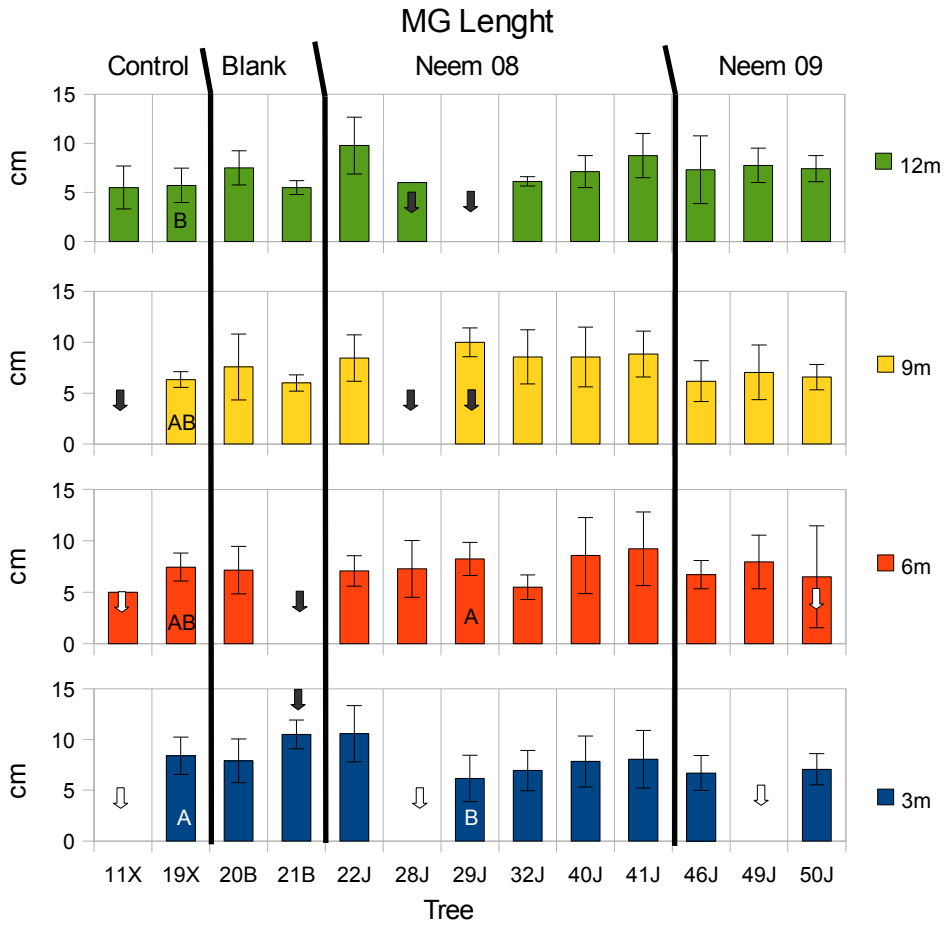


Fig.16: Mother gallery length (cm) (MG length) at different tree heights and treatments ($\dot{x}$, SD). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Log segments marked either with white arrows (low infestation), or black arrows (intensive maturation feeding) had breeding systems $n \leq 3$ and could not be used for statistical analysis. Significant differences ($P<0.05$) between tree heights of the same tree are marked with different capital letters (A, B, AB).

3.1.4. Mother gallery length beyond last established larval gallery

Mother gallery length beyond the last established larval gallery did not indicate any effects of the various treatments on oviposition and regeneration feeding of female beetles. (Fig.17).

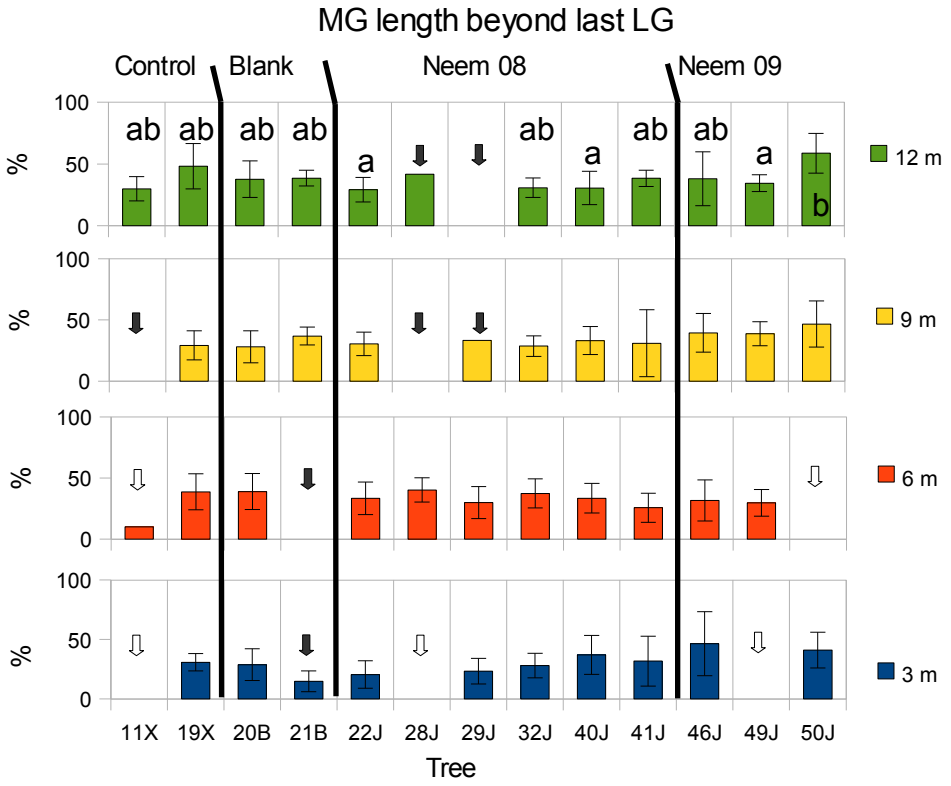


Fig.17: Proportion of mother gallery beyond last established larval gallery in percent of total mother gallery length (MG length beyond last LG) at different tree heights and treatments ($\dot{x}$, SD). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Log segments marked with white arrows (due to low infestation) or black arrows (due to intensive maturation feeding) had n≤3 breeding systems and could not be used for statistical analysis. Significant differences ($P<0.05$) between the various trees at the same height are marked with different lower case letters (a, b, ab).

3.1.5. Number of eggs and larval galleries per mother gallery

The number of eggs per mother gallery (Eggs/MG) was evaluated in order to check the effect of the various treatments on the fecundity of female beetles. In relation to the number of larval galleries per mother gallery (LG/MG) these data were used for further calculation of egg mortality.

One control tree (19X) showed a higher number of eggs per mother gallery at 3 m compared to 12 m and in one of the Neem 08 trees (32J) it was higher in 9 m than in 6 m ($P<0.05$)(Tab.2). However, no significant differences were found between the individual trees or treatments.

Although differences were not always significant, the number of larval galleries in two of three Neem 09 injected trees (46J and 50J) was lower than in all other trees, indicating that less larvae survived than in Neem 08 and control trees (Tab.3).

Tab.2: Number of eggs per mother gallery (Eggs/MG) at different tree heights and treatments, ($\dot{x}$, SD, n). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Log segments marked with "-" due to damaging by maturation feeding had n≤3 breeding systems possible to analyse and therefore could not be used for statistical analysis. Significant differences (P<0.05) between tree heights of the same tree are marked with different capital letters (A, B, AB).

Eggs/ MG		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
12m	$\dot{x}$	47,0	26,0	34,0	31,5	49,1	28,0		33,5	38,0	40,2	34,2	42,8	35,5
	SD	25,2	7,4	18,5	7,0	24,5			13,1	13,1	13,5	18,2	15,6	8,3
	n	3	7	3	4	9	1	n/a	4	9	6	11	8	13
			B				**-**	**-**	**AB**					
9m	$\dot{x}$		29,7	38,4	30,4	45,9		46,0	44,6	38,6	50,0	34,8	38,3	30,7
	SD		7,8	8,1	5,7	17,4		5,7	11,1	18,0	15,1	11,1	17,1	11,5
	n	n/a	3	7	5	10	n/a	2	9	9	3	12	12	7
		-	**AB**				**-**	**-**	**A**					
6m	$\dot{x}$	39,0	38,8	38,1		31,7	31,3	37,9	27,3	42,1	43,6	34,9	41,3	40,0
	SD		7,1	14,6		7,4	10,0	12,9	9,0	20,8	23,6	13,1	17,7	5,7
	n	1	9	10	n/a	7	9	8	8	7	9	12	10	2
		-	**AB**		**-**				**B**					**-**
3m	$\dot{x}$		40,1	40,0	59,5	50,9		36,1	36,4	45,7	38,0	34,0		40,1
	SD		10,8	12,0	16,3	20,6		13,5	10,6	21,3	16,0	9,8		18,9
	n	0	9	10	2	8	0	9	9	6	17	10	0	7
		-	**A**		**-**		**-**		**AB**				**-**	

Tab.3: Number of larval galleries per mother gallery (LG/MG) at different tree heights and treatments($\dot{x}$, SD, n). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Log segments marked with "-" due to damaging by maturation feeding had n≤3 breeding systems possible to analyse and therefore could not be used for statistical analysis. Significant differences (P<0.05) between tree heights of the same tree are marked with different capital letters (A, B, AB), significant differences between the various trees at the same height are marked with different lower case letters (a, b, ab).

LG/ MG		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
12m	$\dot{x}$	36,3	22,1	31,3	28,0	43,1	26,0		27,8	29,1	31,0	5,9	27,6	20,5
	SD	17,9	7,4	15,7	6,1	24,0			10,7	11,9	9,9	6,3	16,7	14,2
	n	3	7	3	4	9	1	n/a	4	9	6	11	8	13
		ab	**a**	**ab**	**ab**	**ab**	**-**	**-**	**ab**	**a**	**AB a**	**b**	**ab**	**ab**
9m	$\dot{x}$		23,7	31,9	26,0	37,3		43,0	41,2	32,6	40,0	11,4	20,8	9,1
	SD		9,9	8,5	3,8	17,9		4,2	10,2	16,1	12,5	12,8	17,4	4,6
	n	n/a	3	7	5	10	n/a	2	9	9	3	12	12	7
		-	**ab**	**ab**	**ab**	**a**	**-**	**-**	**a**	**ab**	**A ab**	**b**	**ab**	**b**
6m	$\dot{x}$	39,0	30,9	25,3		25,9	21,6	27,4	25,0	21,1	19,6	8,6	26,0	27,5
	SD		11,6	13,7		6,6	7,5	10,2	8,4	7,2	13,1	10,7	15,9	0,7
	n	1	9	10	n/a	7	9	8	8	7	9	12	10	2
		-	**a**	**ab**	**-**	**ab**	**ab**	**ab**	**ab**	**ab**	**AB ab**	**b**	**ab**	**-**
3m	$\dot{x}$		25,9	29,2	50,0	34,0		24,9	32,1	26,2	18,3	14,9		17,9
	SD		9,6	14,5	26,9	15,6		7,1	9,0	17,1	10,2	12,2		15,9
	n	0	9	10	2	8	0	9	9	6	17	10	0	7
		-			**-**		**-**				**B**		**-**	

3.1.6. Egg density

To examine possible effects of Neem treatment on continuous egg production and oviposition of female beetles, the number of eggs per cm mother gallery was counted .

There was no indication that egg density in blank, Neem 08 and Neem 09 treated trees was lower than in control trees. Differences between the various tree heights were only found in one Neem 08 tree (29J). Here, the egg density was significantly higher at 3 m than at 6 m ($P<0.05$) (Fig.18).

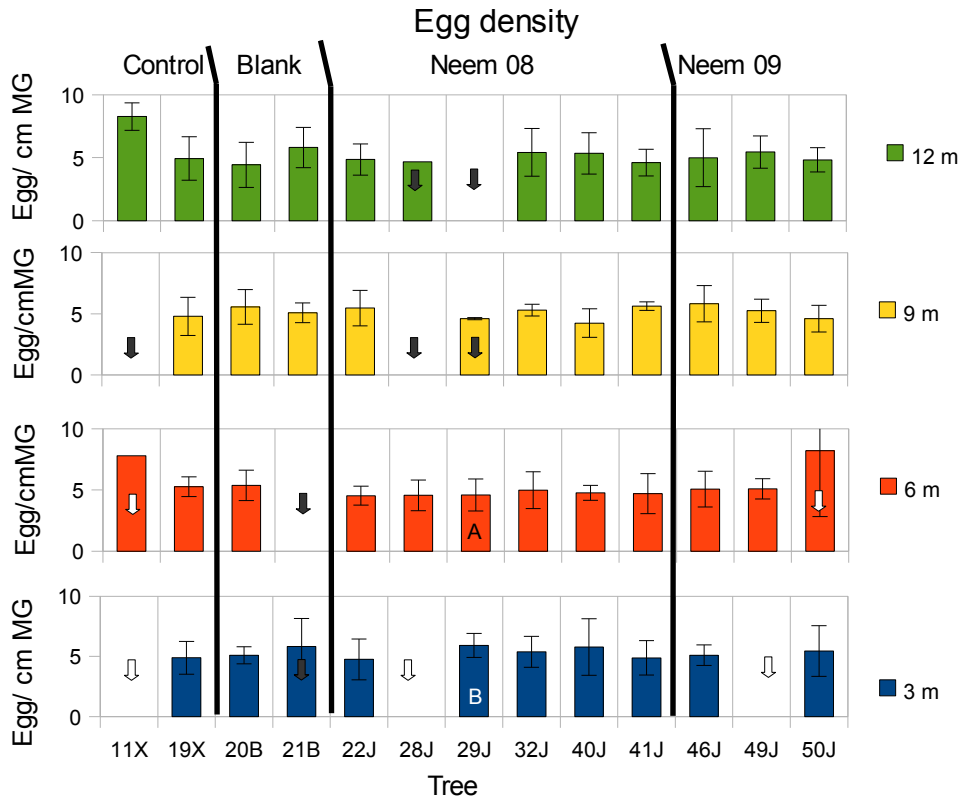


Fig.18: Egg density per cm mother gallery (Egg /cm MG) at different tree heights and treatments ($\bar{x}$, SD). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Log segments marked with white arrows (due to low infestation) or black arrows (due to intensive maturation feeding) had $n \leq 3$ breeding systems and could not be used for statistical analysis. Significant differences ($P<0.05$) between tree heights of the same tree are marked with different capital letters (A, B, AB).

3.1.7. Egg mortality

Egg mortality of one Neem 09 tree (46J) at the upper tree heights (6 m, 9 m and 12 m) was clearly higher than in Neem 08, blank and control trees at the same height ($P<0.05$). In the other Neem 09 trees (49J and 50J) egg mortality was also elevated (although not significantly) compared to the control and blank treated trees. This indicated a systemic effect of Neem stem-injection in at least one tree treated two month earlier (Neem 09 trees).

Furthermore, two Neem 08 trees (40J and 41J) showed a tendency of higher egg mortality at the lower tree heights compared to the other Neem 08, blank and control trees at the same tree height (Fig.19).

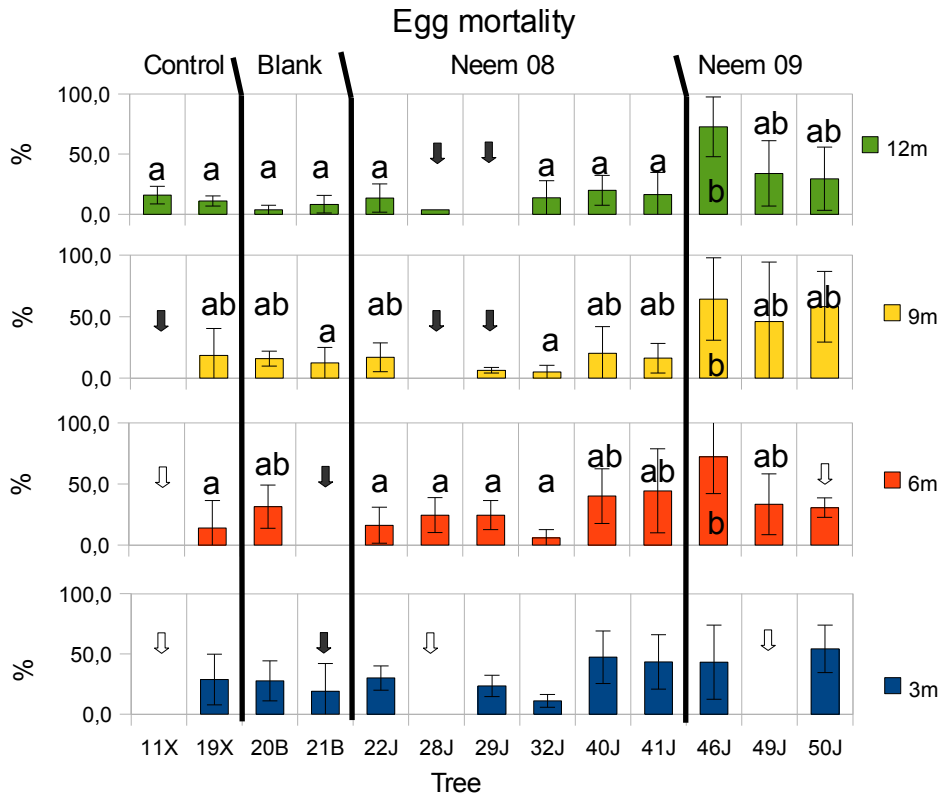


Fig.19: Egg mortality (%) at different tree heights and treatments ($\bar{x}$, SD). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Log segments marked with white arrows (due to low infestation) or black arrows (due to intensive maturation feeding) had $n \leq 3$ breeding systems and could not be used for statistical analysis. Significant differences ($P<0.05$) between the various trees at the same height are marked with different lower case letters (a, b, ab).

3.1.8. Larval mortality

The number of incomplete larval galleries due to larval death was related to the total number of larval galleries to examine whether the various treatments had effects on larval mortality. In many trees and tree heights it was not possible to classify successful and unsuccessful larval galleries, because in some breeding systems they were destroyed due to feeding activity of F1-beetles (maturation feeding). Therefore, the sample size was much lower than for other breeding parameters.

Again, Neem 09 tree 46J showed – like in egg mortality - lower number of successful larval galleries than Neem 08, blank treatment or untreated control trees (Tab.4). The calculation of the larval mortality showed similar results. Larval mortality was significantly higher in Neem 09 tree (46J) at 12 m compared to one Neem 08, one Neem 09 and the control tree (19X) ($P<0.05$). Neem 08 trees 40J and 41J showed also a trend for higher larval mortality at 3 and 6 m compared to other Neem 08, blank or control trees (Fig.20).

Like it was shown for emergence of beetles and egg mortality, stem-injection of Neem into the tree reduced progeny development by a systemic effect but effects varied between trees and tree .

Tab.4: Number of incomplete larval galleries per mother gallery (incomplete LG / MG) of log segments from trees of various treatments and tree heights ($\bar{x}$, SD, n). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09).Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Log segments marked with "-" had n≤3 breeding systems and could not be used for statistical analysis. Significant differences ($P<0.05$) between the tree heights of the various trees are marked with different lower case letters (a, b, ab).

aborted LG		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
12m	$\bar{x}$		0,3			6,0				2,0		5,6	3,7	3,6
	SD		0,6			8,5				2,8		4,0	3,5	2,8
	n	n/a	3	n/a	n/a	2	n/a	n/a	n/a	2	n/a	7	3	8
		-		-	-	-	-	-	-	-	-			
9m	$\bar{x}$			0,0	1,3	3,0			0,0	0,0	12,0	6,8	3,6	3,5
	SD				1,5	3,8			0,0			3,2	4,2	5,1
	n	n/a	n/a	1	3	4	n/a	n/a	2	1	1	6	5	4
	-	-	-	-	**ab**	**b**	-	-	-	-	-	**a**	**a**	**a**
6m	$\bar{x}$	0,0	1,5	0,0			3,5	0,0	2,0	8,3	12,7	7,3	4,6	4,0
	SD		2,1	0,0			4,7		2,6	5,6	11,0	5,6	4,6	2,8
	n	1	2	4	n/a	n/a	4	1	3	4	3	6	5	2
		-	-	-	-	-		-						-
3m	$\bar{x}$		8,4	7,5		0,0		0,7	1,0	6,7	9,2	5,6		2,0
	SD		6,6	7,8				1,2	2,0	9,0	8,5	4,0		2,0
	n	0	7	6	0	1	0	3	4	3	10	7	0	5
		-	**ab**	**ab**	-	-	-	**ab**	**b**	**ab**	**a**	**a**	-	**ab**

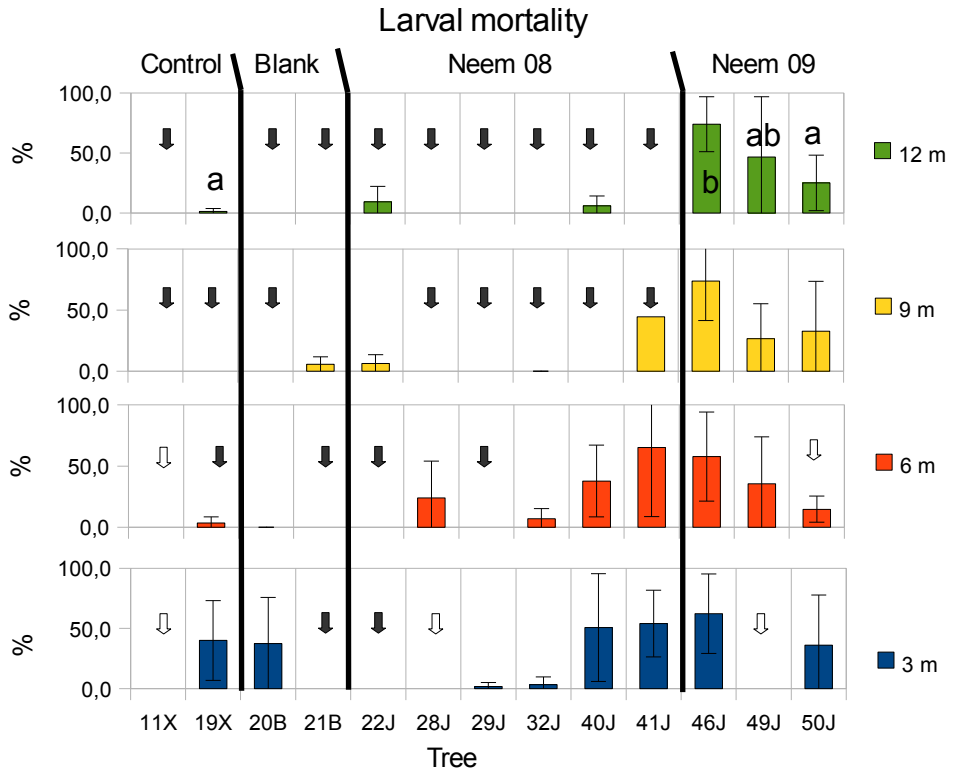


Fig.20: Larval mortality (%) at different tree heights and treatments ($\dot{x}$, SD). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Log segments marked with white arrows (due to low infestation) or black arrows (due to intensive maturation feeding) had $n \leq 3$ breeding systems and could not be used for statistical analysis. Significant differences ($P<0.05$) between the tree heights of the various trees are marked with different lower case letters (a, b, ab).

3.2. Breeding activity of beetles in untreated logs

After emergence of parental and F1-beetles from log segments of different treatments (Neem 08, Neem 09, blank and untreated control) the beetles were mounted with capsular pits on untreated spruce logs in order to check their ability to establish new broods. However, this was not possible for all treated trees, since the number of emerging beetles from blank tree 21B and Neem 09 trees 46J and 50J was too low to set up the breeding experiments. Only beetles from one Neem 09 tree (49J) could be tested.

3.2.1. Breeding willingness

The breeding willingness of parental and F1-beetles was determined by the number of male and female beetles that successfully established nuptial chambers and mother galleries, respectively. This rate was compared to the number of beetles that did not enter the bark or established only feeding galleries.

3.2.1.1. *Parental beetles*

Due to statistical differences in breeding success of beetles from same tree between experimental logs felled in November 2008 (Nov 08 logs) and those in April 2009 (Apr 09 logs), these data were listed separately for each felling date (Fig.21 a,b).

The breeding success of females differed significantly from different felling dates of the logs used in the experiment. However, compared to control females within the same felling date, neither blank nor Neem 08 treatment showed an effect on the willingness of female beetles to enter the log segments and establish breeding systems (Fig.21 b). Low willingness of male beetles to enter the bark and establish nuptial chambers compared to males from the control tree was only observed for beetles from the blank treated tree on logs felled in November 08 (Fig.21 a).

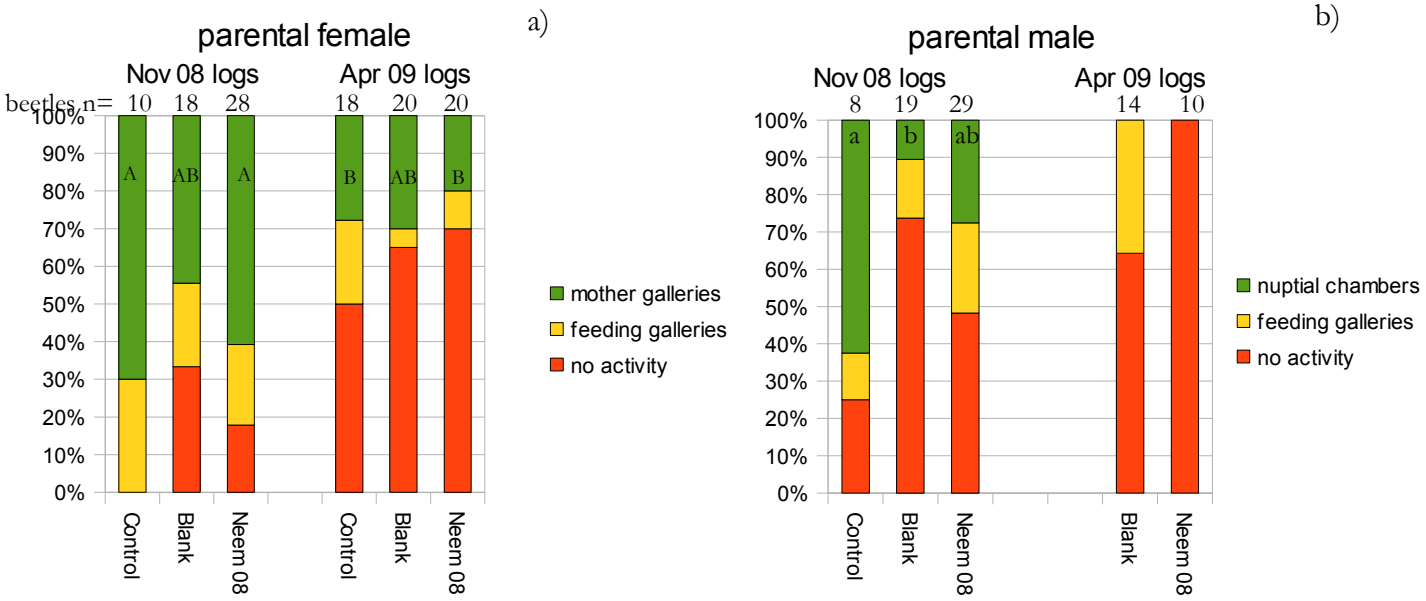


Fig.21 a,b: Percentage of female (a) and male (b) parental beetles establishing mother galleries, nuptial chambers or feeding galleries, as well as percentage of beetles not entering the bark on logs felled in November 2008 (Nov 08 logs) and April 2009 (Apr 09 logs). Beetles mounted to the untreated experimental logs emerged from forest trees injected with solvent solution (blank) in 2008, trees injected with Neem in 2008 (Neem 08) or from untreated control trees (control). Different capital letters show significant differences ($P< 0.05$ χ^2) in mother galleries for the same treatment between experimental logs of different felling dates. Different lower case letters show significant differences in nuptial chambers between the various treatments in Nov 08 logs .

3.2.1.2. F1-beetles

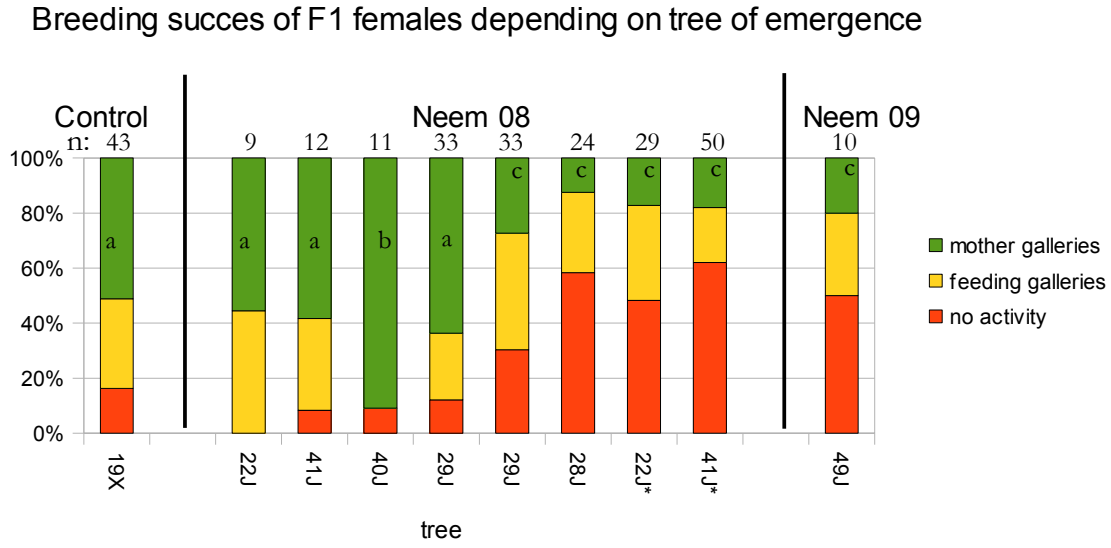
While parental beetles were mounted to logs from two trees that were each felled on a different day, F1-beetles were mounted to logs of two different trees felled on the same day.

The breeding success of female beetles emerging from the Neem 09 treated tree was lower than those of control beetles. Given that logs of the same tree were used for the experiment, number of mother galleries varied considerably between beetles of different Neem 2008 trees (Fig.22a). This pattern is not reflected by the mortality of progeny mentioned earlier in this study and does not imply different concentrations of Neem in 2008 treated trees.

The logs from different trees seemed to have effects on the breeding success of beetles, given that beetles emerged from the same Neem 2008 trees (22J and 41J).(Fig.22a).

The rate of nuptial chambers did not indicate any effects of Neem 09 nor Neem 08 treatment on male beetles and their activity did not correspond to those of female beetles (Fig.22b).

a)



b)

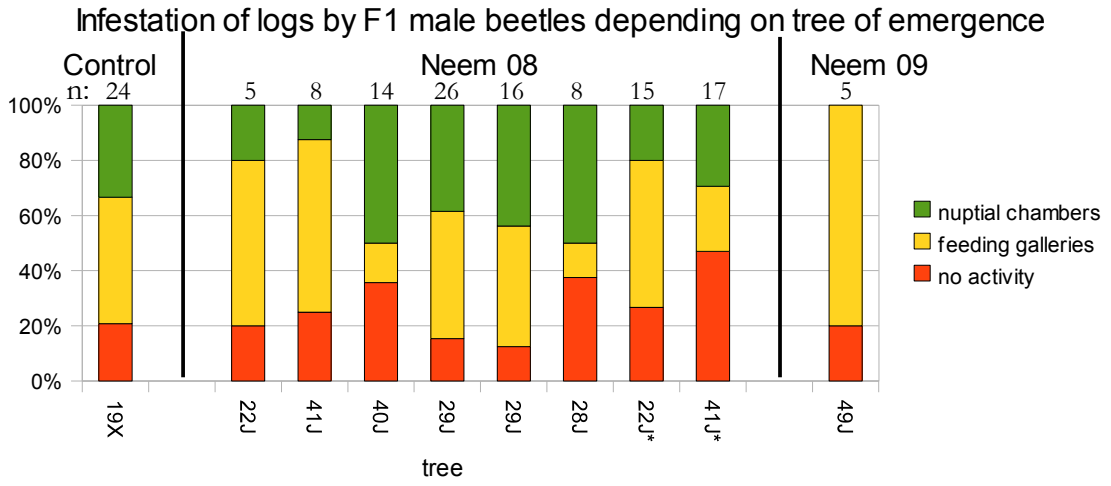


Fig.22 a,b: Percentage of female (a) and male (b) F1-beetles establishing mother galleries, nuptial chambers or feeding galleries, as well as percentage of beetles not entering the bark after mounting to the untreated logs. Beetles emerged from log segments of the untreated control (tree 19X), Neem-injected in 2008 (Neem 08 trees 22J, 28J, 29J, 40J and 41J) and Neem-injected in 2009 (Neem 09 tree 49J). Beetles mounted to logs obtained from a different tree are indicated by"*". Beetles from Neem 08 tree 29J differed significantly between logs and therefore are shown separately. Different lower case letters show statistical differences for number of mother galleries. No statistical differences for nuptial chambers with statistical comparable data was observed ($P< 0.05$ χ^2).

3.2.2. Analysis of breeding systems

The breeding systems of parental (sister brood) and F1 females (F2-brood) from Neem and blank-injected trees were checked for the following parameters in the untreated logs: MG length, eggs per MG, egg density, LG per MG, egg mortality and larval mortality. Again, the harvesting date of the offered logs had a significant influence on the recorded parameters. Therefore, the results are presented separately.

3.2.2.1. Parental beetles

The breeding systems of Neem 08 parental beetles did not differ from control or blank treated trees in either of the felling dates. Therefore, Neem treatment had no influence on female parental beetles and their progeny in establishing new breeding systems one year after injection. Interestingly, females from the blank treatment laid significantly less eggs than control beetles in freshly offered logs felled in November 2008. Furthermore, the breeding systems from these beetles showed shorter mother gallery lengths, less number of eggs, lower egg density and less number of larval galleries than those of the same treatment mounted to the logs felled in April 2009. (Fig.23-28 a,b).

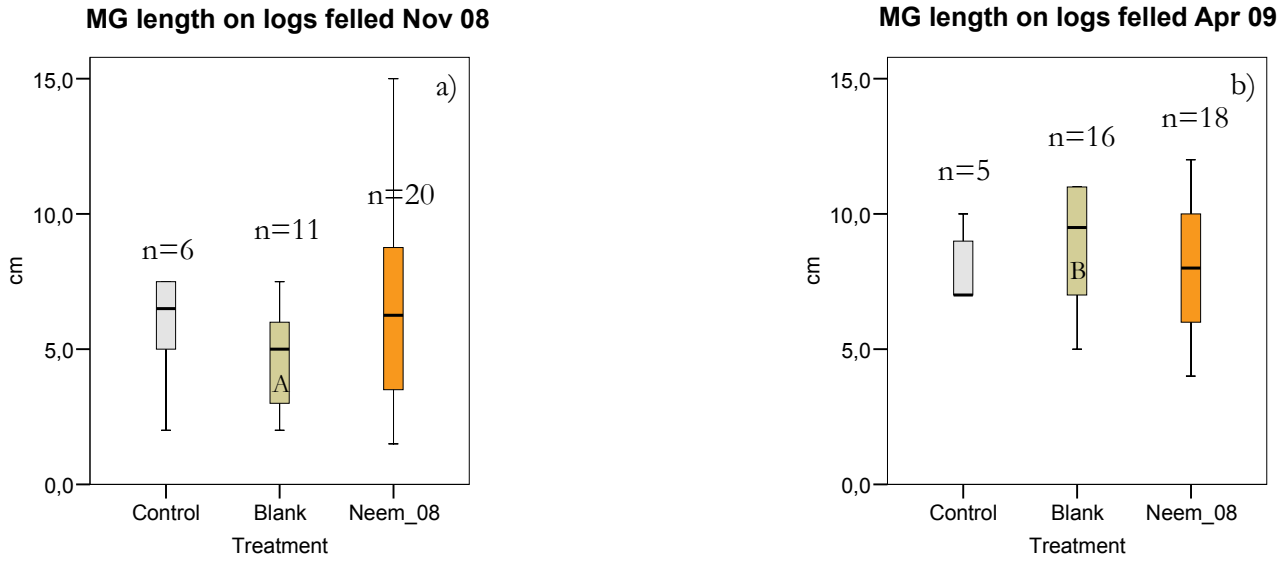


Fig.23 a,b: Mother gallery length in cm (MG length cm) of sister brood breeding systems after mounting parental beetles with capsules to untreated experimental logs felled in November 2008 (Nov 08) (a) or in April 2009 (Apr 09) (b). Parental beetles had emerged from log segments of one untreated control tree (control), one tree injected with solvent solution in 2008 (blank) and one tree injected with Neem in 2008 (Neem 08). Capital letters indicate significant differences between same treatments on logs of different felling dates (P<0.05).

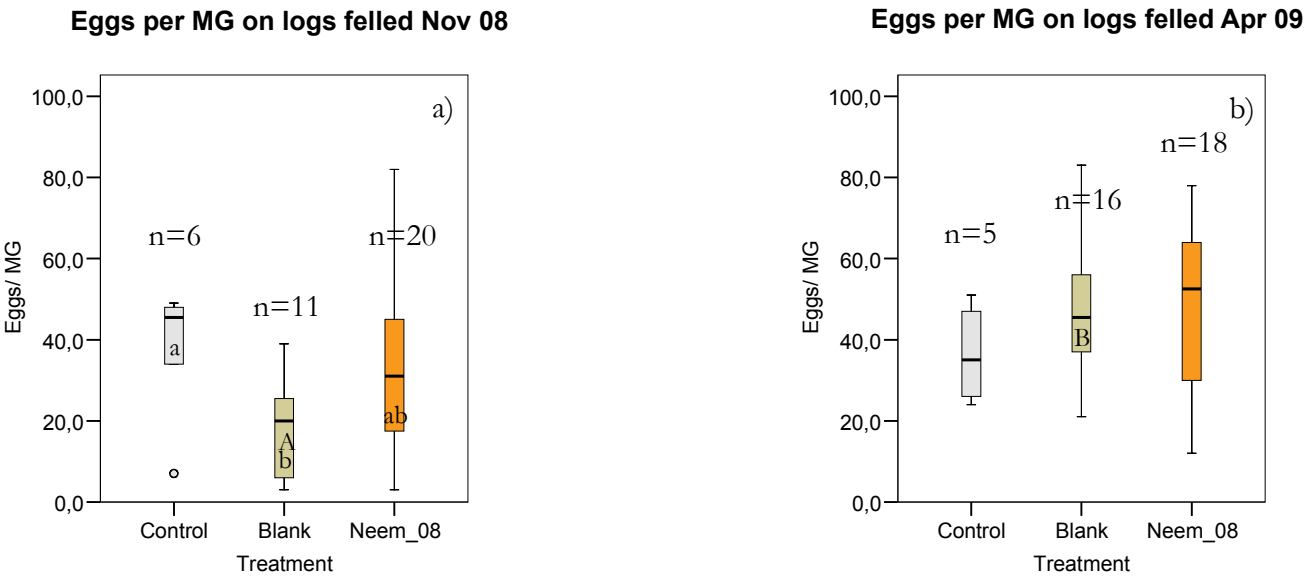


Fig.24 a,b: Number of eggs per mother gallery (Eggs/ MG) of sister brood breeding systems after mounting parental beetles with capsules to untreated experimental logs felled in November 2008 (Nov 08) (a) or in April 2009 (Apr 09) (b). Parental beetles had emerged from log segments of one untreated control tree (control), one tree injected with solvent solution in 2008 (blank) and one tree injected with Neem in 2008 (Neem 08). Lower case letters indicate significant differences between treatments on logs of the same felling date. Capital letters indicate significant differences between same treatments on logs of different felling dates (P<0.05).

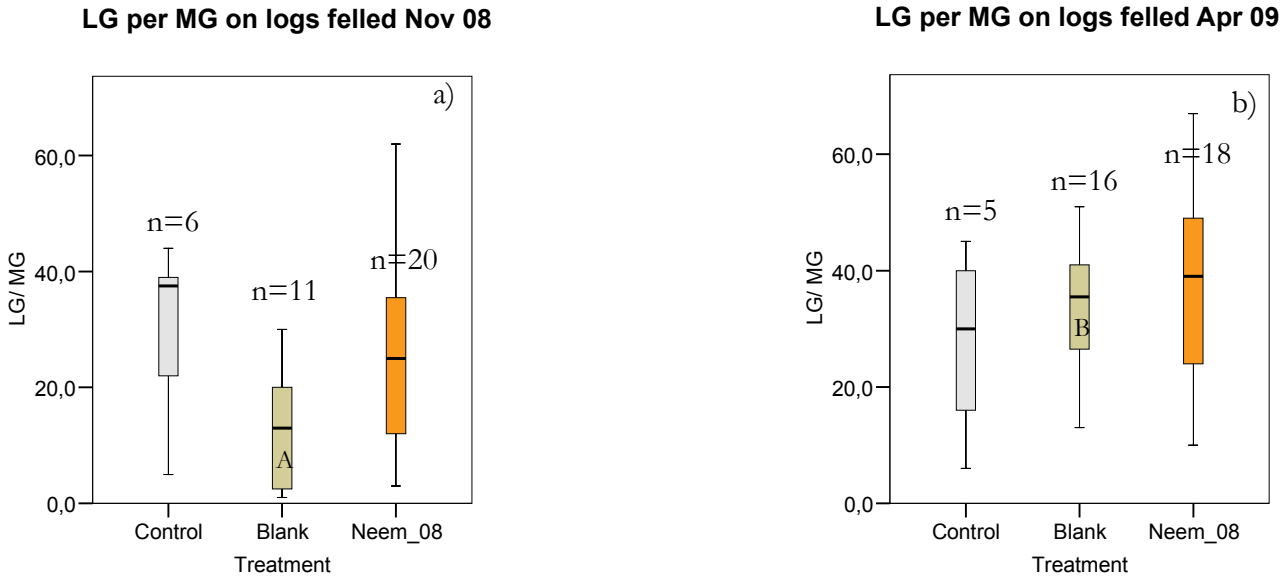


Fig.25 a,b: Number of larval galleries (LG/ MG) of sister brood breeding systems after mounting parental beetles with capsules to untreated experimental logs felled in November 2008 (Nov 08) (a) or in April 2009 (Apr 09) (b). Parental beetles had emerged from log segments of one untreated control tree (control), one tree injected with solvent solution in 2008 (blank) and one tree injected with Neem in 2008 (Neem 08). Capital letters indicate significant differences between same treatments on logs of different felling dates (P<0.05).

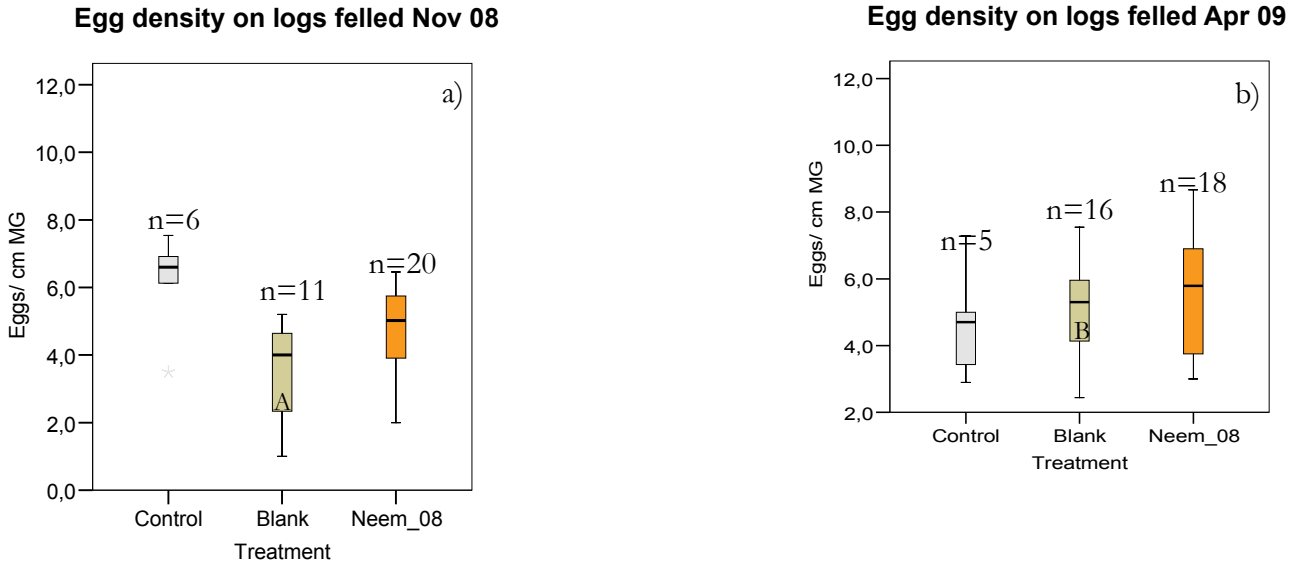


Fig.26 a,b: Egg density (n/ dm²) of sister brood breeding systems after mounting parental beetles with capsules to untreated experimental logs felled in November 2008 (Nov 08) (a) or in April 2009 (Apr 09) (b). Parental beetles had emerged from log segments of one untreated control tree (control), one tree injected with solvent solution in 2008 (blank) and one tree injected with Neem in 2008 (Neem 08). Capital letters indicate significant differences between same treatments on logs of different felling dates (P<0.05).

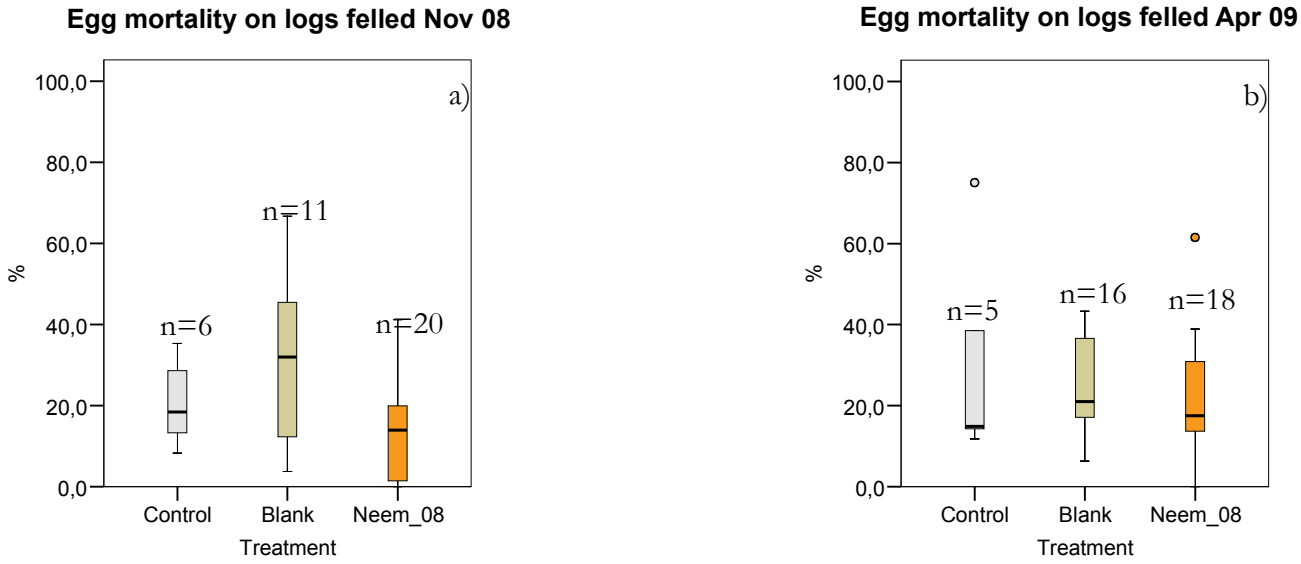


Fig.27 a,b: Egg mortality (%) of sister brood breeding systems after mounting parental beetles with capsules to untreated experimental logs felled in November 2008 (Nov 08) (a) or in April 2009 (Apr 09) (b). Parental beetles had emerged from log segments of one untreated control tree (control), one tree injected with solvent solution in 2008 (blank) and one tree injected with Neem in 2008 (Neem 08).

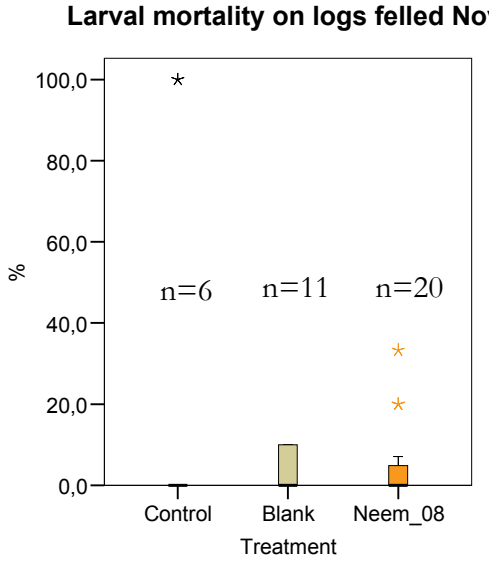
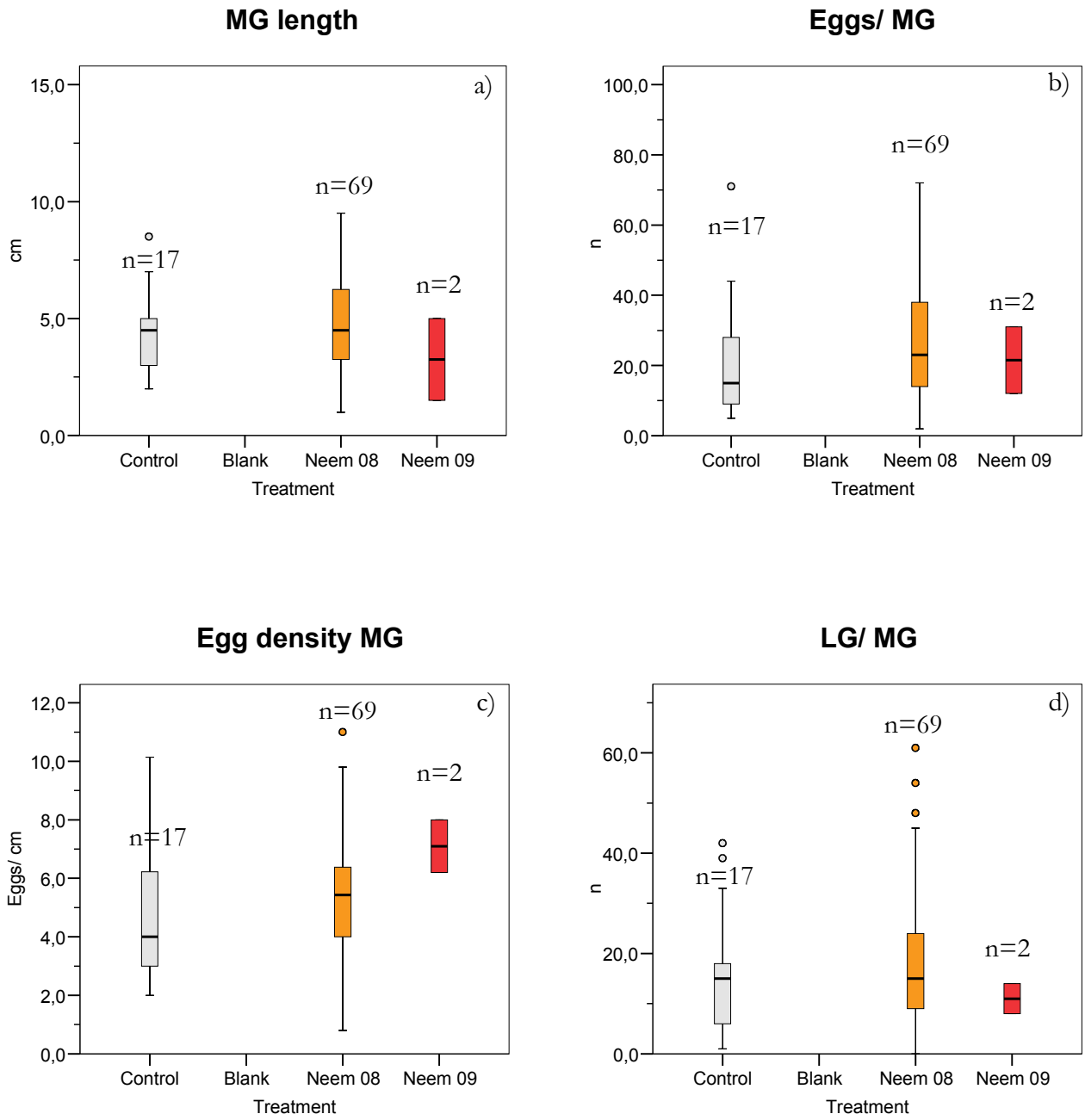


Fig.28: Larval mortality (%) of sister brood breeding systems after mounting parental beetles with capsules to untreated experimental logs (felled in November 2008 (Nov 08) (a) or in April 2009 (Apr 09) (b). Parental beetles had emerged from log segments of one untreated control tree (control), one tree injected with solvent solution in 2008 (blank) and one tree injected with Neem in 2008 (Neem 08). Development time for larvae in logs in April 2009 (Apr 09) was too short to evaluate larval mortality.

3.2.2.2. F1-beetles

The breeding parameters of F1-beetles from Neem 09 trees could not be statistically analysed since only two breeding systems were established. Number of eggs and number of larval galleries of these two breeding systems were in the range of those in control and Neem 08 treatment. No differences for the breeding parameters were found between control and Neem 08 treatment (Fig.29a,b,c,d,e,f).



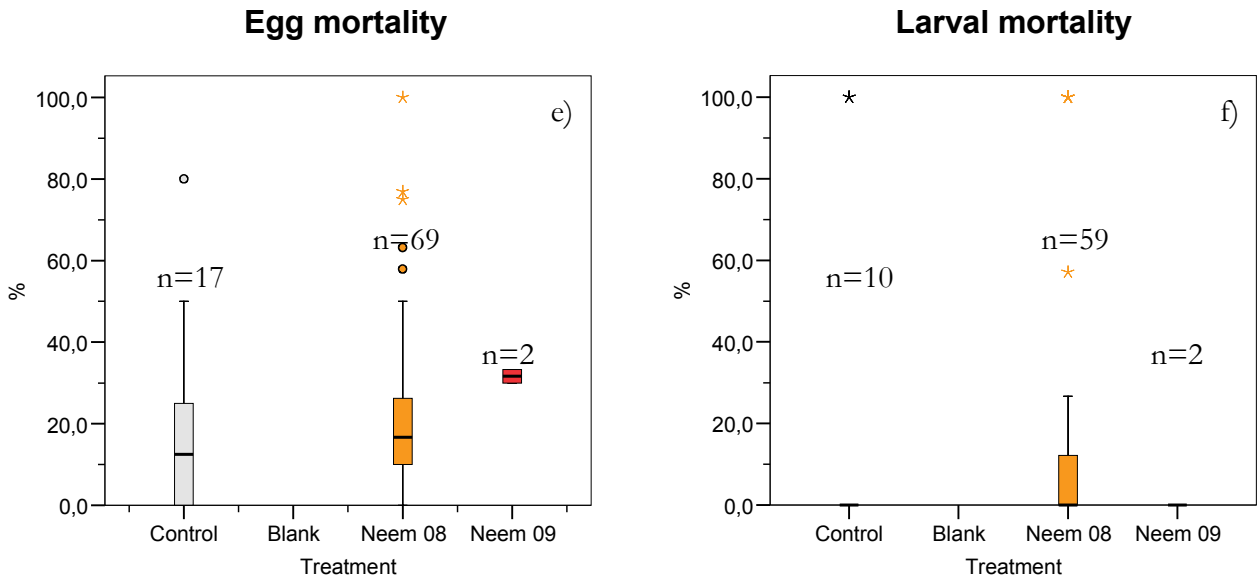


Fig.29 a,b,c,d,e,f: Mother gallery length cm (MG length cm) (a), Eggs per mother gallery (Eggs/ MG) (b), Egg density (Eggs/cm MG) (c), Larval galleries per mother gallery (LG/MG) (d), Egg mortality in percent (%) (e) and larval mortality in percent (%) (f) of F2-brood breeding systems (BS) after mounting of filial 1 (F1) beetles with capsules to untreated experimental logs. Beetles had emerged from log segments of one untreated control tree (control), two trees injected with Neem in 2008 (Neem 08) and one tree injected with Neem in 2009 (Neem 09 tree 49J). "n" is indicating the number of breeding systems that were successfully established by F1-beetles of the various treatments. Number of breeding systems of beetles from Neem 09 trees was n≤3 and therefore could not be used for statistical analysis (P<0.05).

3.3. Maturity of ovarioles of emerged female beetles

To check possible effects of Neem stem-injection on the reproductive activity of female parental and F1-beetles breeding or developing in Neem trees, the area of their dissected ovarioles was measured and the presence of a corpus luteum (yellow follicle cell remnants at the basis of the ovarioles and the oviduct), was recorded indicating recent oviposition by parental female beetles (Fig.30).

The presence of the corpus luteum in ovarioles of beetles depends on the date of emergence. Beetles that emerged between May 24 and June 1 from either control, blank or Neem 08 treated trees always showed a corpus luteum. After a short transition period, no corpus luteum was observed in ovarioles of beetles that emerged after June 7 (Tab.5). This indicates that parental beetles re-emerged at the beginning of the experiment, while later only F1-beetles without previous oviposition emerged from the log segments.

Tab.5: Number of emerged female beetles from one untreated control tree (control), one tree injected with solvent solution in 2008 (blank) and one tree injected with Neem in 2008 (Neem 08) showing ovaries with corpus luteum.

Emergence date	Corpus luteum	Control	Blank	Neem 08
May 24 to June 1	yes	1	4	3
June 2 to Juni 6	yes	0	2	1
	no	1	2	0
June 7 and June 8	no	3	5	4
June 24 to July 5	no	5	0	22

The size of parental and F1-female ovaries were not different between control, blank, and Neem 08 treatment. Therefore, it can be concluded that the Neem treatment of trees had no influence on the reproductive activity of *I. typographus* one year later. No differences were found between parental and F1-beetles. Both beetle generations had fully developed ovaries (Fig.30).

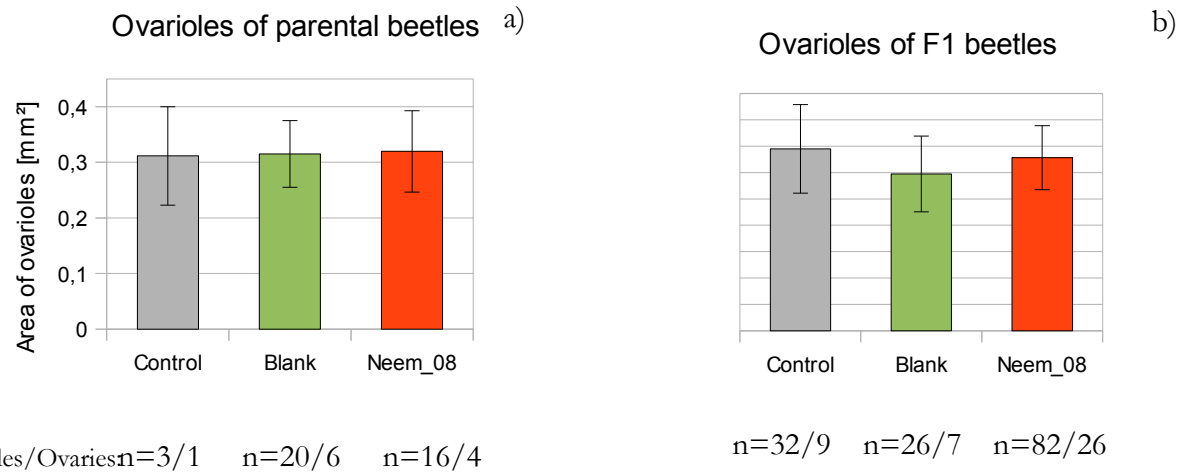


Fig.30 a,b: Area of female ovarioles/ovaries [mm²] of parental beetles (with corpus luteum) (a) and F1-beetles (without corpus luteum) (b). Beetles emerged from untreated control trees (control), trees injected with blank solvent solution in 2008 (blank) and trees injected with Neem in 2008 (Neem 08).

3.4. Natural enemies of *I. typographus*

The most prevalent natural enemies which emerged from trees infested with bark beetles were the dipteran predator on *I.typographus* larvae, *Medetera sp.* (Dolichopodidae), the endoparasitic braconid wasp of adult beetles, *Ropalophorus clavicornis* (Braconidae) and three ectoparasitic wasps, *Coeloides bostrichorum* (Braconidae), *Rhopalicus sp.* and *Roptrocerus sp.* (Pteromalidae) feeding on bark beetle larvae. Two ectoparasitoids of bark beetle larvae, *Dendrosoter mittendorfii* (Braconidae), *Rhopalicus tutella* (Pteromalidae), and the endoparasitoid *Tomicobia seitneri* (Pteromalidae) of adult beetles occurred only sporadically (less than ten individuals). The number of ecto-and endoparasitoid species was not lower in Neem 09 trees compared to blank and control trees (Tab.6).

In total, four different species emerged from beetles developing in Neem 09 trees. The highest number of predator and parasitoid species were found in two of the Neem 08 treated trees (seven and five species). No more than two species emerged from Neem 09 treated trees and one Neem 08 tree. The occurrence of the larval ectoparasitoid Rhopalicus sp. seemed to be limited to the area in the south-west of the experimental site (Fig.3).

Tab.6: Occurrence of natural enemies (more than ten individuals per species and segment : x; less than 10 individuals per segment: (x)) that emerged from combined log segments of lower (3 m, 6 m) and upper tree heights (9 m, 12 m) with different treatments. Tree 19X was untreated (control), tree 20B was injected with solvent solution in 2009 (blank 09), trees 22J- 41J were injected with Neem in 2008 (Neem 08) and trees 46J-50J were injected with Neem in 2009 (Neem 09).

Treatment	Control	Blank	Neem 08					Neem 09		
Tree	19X	20B	22J	28J	29J	40J	41J	46J	49J	50J
Species										
Dolichopodidae										
Medetera sp. (predator)	x	x	x	x		x	x	x		
Pteromalidae										
Rhopalicus sp. (larval ectoparasitoid)			x	x	x		x			
Rhopalicus tutela (larval ectoparasitoid)			(x)							
Tomicobia seitneri (adult endoparasitoid)				(x)						(x)
Roptrocerus sp. (larval ectoparasitoid)			x	x	x	x	x		x	x
Braconidae										
Ropalophorus clavicornis (adult endoparasitoid)	x	x	x	x		x	x	x		
Coeloides bostrichorum (larval ectoparasitoid)	x	x	x							
Dendrosoter mittendorfii (larval ectoparasitoid)			(x)							

4. Discussion

The objective of this study was to examine the effects of stem-injected Neem solution on the reproductive activity of parental and both filial generations (F1 and F2) of *I. typographus*. In order to test the persistence of Neem in the trees, trees were injected either one year (in 2008) or one month (in 2009) prior to bark beetle infestation.

Principally, the reproductive activity of attacking beetles on Neem-treated logs might be inhibited due to repellent and anti-feeding effects by this agent. Repellent effects of Neem after topical spraying on conifer trees were observed for the large pine weevil, *Hylobius abietis* (Thacker et al., 2003; Sibul et al., 2009) and several other Coleopteran species that feed on agricultural plants and storage products (Kaethner, 1992; Palaniswamy and Wise, 1994; Xie et al., 1995). Neem-induced repellent and anti-feeding effects were also reported for *I. typographus* in some experiments and under certain circumstances: The number of nuptial chambers and length of mother galleries was reduced after a topical spray consisting of an undetermined concentration of Neem that was applied to the logs for an unspecified amount of time (Kreutz, 2007). These properties of Neem could be caused by the repellent effects on male beetles combined with anti-feeding behaviours and/or reduced oviposition of females. Shorter mother gallery length indicating feeding deterrence was noted when logs were sprayed two weeks before infestation by Weber (2011). However, in the same study no repellent effects on male and female beetles entering the bark were observed (Weber, 2011). This was also confirmed with Neem sprayed logs and Neem-injected trees in field studies (Kolev, 2011). Similarly, results indicating no repellency of Neem stem-injection were found for other phloem feeding bark beetles after stem injection (Naumann et al., 1994; Duthie-Holt et al., 1999). Especially this method might reduce possible existing deterrence effects for attacking beetles.

One of the main factors possibly reducing reproduction of beetles is an effect of Neem which causes decreasing egg production rates in female beetles. This effect has been documented for several other insect species: After topical application of Azadirachtin solutions to larvae and adults, lower oviposition rate and decreased size of ovarioles was observed in the grasshopper *Heteracris littoralis* (Ghazawi et al., 2007). Reduced ecdysteroid levels were reported to be responsible for low reproduction activity of newly hatched female earwigs (*Labidura riparia)* that were previously injected with Azadirachtin (Sayah et al., 1998). Oviposition by two cockchafer species *Melolontha hippocastani* and *M. melolontha* as well as the Colorado potato beetle (*Leptinotarsa decemlineata)* decreased after feeding on Neem-treated plants (Kaethner, 1991, 1992). Neem treatment affecting oviposition of F1-beetles were found for the cowpea bruchid beetle (*Callosobruchus maculatus)* which laid significantly less eggs after feeding on Neem-treated chickpeas (*Cicer arietinum)* compared to beetles that had developed on untreated peas (Elhag, 2000).

The effects of Neem treatment on the ovarian development could also become manifested by decreased emergence and decreased willingness of beetles to establish new breeding systems.

As far as the data available shows, the number of re-emerging parental beetles and their willingness to establish breeding systems seem to be not affected when Neem was injected into the test trees one year earlier (in 2008). A different experiment observed that Neem treatment did not reduce the ovarian maturity of *I. typographus* parental female beetles that re-emerged from topically treated logs (Weber, 2011). The number of mother galleries established by F1-beetles from trees injected the year before (in 2008) differed to a high degree, which might be due to overpopulation and concurrencethe beetles emerged from. These were heavily damaged by juvenile feeding. The pattern of high and low number of established mother galleries in the untreated logs was neither reflected by the mortality of progeny nor by the size of female gonads in the treated trees the beetles had been collected from.

Recent Neem injection (in 2009) seems to lower the willingness of F1 female beetles to infest untreated trees. Even though breeding performance itself was not reduced, the low total number of successful breeding due to prior mortality and reduced willingness to breed suggests that the emerging F1-generation is not posing major danger for further population outbreaks.

In none of the cases were effects of Neem stem-injection on the activity of male beetles establishing nuptial chambers observed after development in trees treated by Neem-injections.

Neem treatment generally did not affect the oviposition and gonad maturation of *I. typographus*. This is confirmed by the fact that parental beetles establishing breeding systems in trees treated the same year or the year before laid as many eggs as in controls. No indication of Neem influencing ovarian development of *I. typographus* was found after topical application (Weber 2011). Likewise, the stem-injection of Neem into trees one month before attack by beetles had no effects on mother gallery length and egg deposition (Kolev, 2011).

Nevertheless, due to the synovigen type of egg development in females of *I. typographus*, it might be assumed that Neem may have a delayed effect on egg development. However, there was no indication that those beetles which established successfully brood systems in untreated logs were influenced by former Neem treatment. Moreover, no increased mortality was observed for their progeny.

Injection of Neem into trees had no effect on reproduction activity of adult beetles, but was found to cause high mortality during the juvenile development of the beetles. Neem sprayed topically on logs two weeks before infestation induced up to 100% egg- and larval mortality of *I. typographus* (Weber, 2011). However, in my study using the injection method the effects varied between each tree and different heights within the same tree. Thus, this study confirmed the results of Kolev (2011) who made similar observations after stem-injection. The variation suggests that translocation of

Azadirachtin in the tree might be strongly influenced by individual tree physiology and anatomy. Weber (2011) further observed that high mortality of *I. typographus* larvae occurred during the last instars when logs were treated topically two weeks after infestation. Banken and Stark (1997) reported that last instars (4^{th}) of the seven-spot ladybird (*Coccinella septempunctata)* were also to be most susceptible to Neem treatment.

As a consequence of high mortality during progeny development, the emergence rate of F1-beetles from recently treated trees was markedly lower than from controls. In addition, almost no newly developed beetles were found at the inner side of the bark. Both the low emergence and the low number of beetles found in the adult stage supports the former observation that juvenile development was markedly reduced. Earlier experiments proved that Neem stem-injection lead to high mortality of the pine engraver beetle (*Ips pini*) and the emerald-ash borer (*Agrilus planipennis*) on lodgepole pine (*Pinus contorta*) and green ash (*Fraxinus pennsylvanica)*, respectively, using similar or lower concentrations of Neem like in my study (Duthie-Holt et al., 1999; McKenzie et al., 2010).

Neem treatment might not only reduce the development of *I. typographus* but also might have unwanted side-effects on natural enemies of the bark beetle. However, stem-injection of Neem into trees did not seem to influence the spectrum of parasitoid or predator species. Since the total number of emerged species was not evaluated, no definitive answer about the effects on the complex of natural enemies of *I. typographus* can be given. Previous reports on the mortality effects of Neem on natural enemies of plant pests showed ambiguous results: When larvae of the whitefly *Bemisia tabaci* and the rice moth *Corcyra cephalonica* fed on leaves treated with low doses of Azadirachtin (below 200 mg/l), the emergence of the larval endoparasitoid *Encarsia sophia* and ectoparasitoid *Habrobracon hebetor* (both Hymenoptera., Braconidae) was not reduced (Aggarwal and Brar, 2006; Adarkwah et al., 2011). However, another study using similar concentration of Neem, systemically transported in cabbage induced significantly higher mortality of the predators *Coccinella septempunctata, Chrysoperla carnea, Episyrphus balteatus* and the endoparasitoid *Diaeretiella rapae* of aphids on this plant than than in controls (Ahmad et al., 2003).

Translocation of Neem in stem-injected trees is yet not fully understood. Azadirachtin has to be transported from the site of injection upward in the xylem or by radial transport via apoplast from the sap wood to the phloem where *I. typographus* is breeding. Heidecke (2006) examined the axial, tangential and radial transport of the staining solution Safranin-O in horse chestnut *(Aesculus hippocastaneum)*, sycamore maple (*Acer pseudoplatanus),* large-leafed linden *(Tilia platyphyllos)* and pedunculate oak *(Quercus robur).* In addition to xylem transport, all studied diffuse-porous trees (*Acer, Tilia, Aesculus*) showed radial transport of more than 4 cm reaching also the phloem parts of the tree (Heidecke, 2006). However, it is not certain whether Neem is transported in trees in the same way as the staining solution

Safranin-O. Transport of Neem in the xylem of conifer trees may be less efficient than in deciduous trees, as conifers possess only tracheids but no trachea like deciduous trees. Trachea transport water and dissolved substances more quickly than the tracheids of the conifers (Heidecke, 2006; Bresinsky et al., 2008). In earlier studies it was shown that Azadirachtin can be transported via the xylem to the foliar parts of trees. Thus, stem-injection of Neem significantly reduced the larval development of the spruce budworm *(Choristoneura fumiferana)* and horse-chestnut leaf miner (*Cameraria ohridella)* feeding on needles of white spruce *(Picea glauca)* and leaves of horse chestnut *(Aesculus hippocastaneum),* respectively (Sundaram et al., 1997; Helson et al., 2001; Pavela and Bárnet, 2005). Traces of Azadirachtin were found in leaves of white ash trees (*Fraxinus americana*) eleven days after stem-injection, but concentration of the compound declined logarithmically during the following 17 days (McKenzie et al., 2010). In this study, it was not examined whether Neem degraded in the needles or was transported downward in the phloem. Testing Neem uptake via root system in 20-month old Norway spruce seedlings showed that concentration was more than five times higher in needles and shots than in the bark and the wood (Sundaram, 1996). Although concentration in the bark might be low, various studies proved that Neem is able to affect bark beetle development in the phloem. Kolev (2011) observed increased egg- and larval mortality as well as reduced emergence as far as 15 m tree height. Reduced emergence was also found for *Ips pini* after stem-injection of Neem into *Pinus contorta* reaching as far as 9 m beyond the point of injection (Duthie-Holt et al., 1999).

For the effectiveness of the stem injection method it is important to consider the persistence of Neem in the tree. Neem that had been injected in Norway spruce trees one year before infestation had no adverse effects on development of *I. typographus* progeny in the majority of the treated trees. Various environmental factors like temperature, water, air and dissolved substances in the tree might be involved in degrading Neem during the course of one year. Kolev (2011) showed that Neem persisted up to four months after stem-injection in spruce trees. Stem-injection of Neem into small (8 cm in diameter) green ash trees *(Fraxinus pennsylvanica)* indicated that injection of the agent was effective to reduce the number of emerging *A. planipennis* one year after injection (McKenzie et al., 2010). Since the live cycle of *A. planipennis* spans over the period of one year (sometimes even two years), it is not clear whether low emergence was caused by high larval mortality shortly after stem-injection or if Neem treatment also affected hatching and emergence of beetles after overwintering. In the same study, the concentration of Azadirachtin in the leaves of the trees degraded by half in a relatively short time of 20 days suggesting that residual time of Neem in the tree may be shorter than one year.

Concerning the use of Neem for spruce bark beetle control, two different methods have been tested so far; the application of Neem via stem-injection of living trees (Kolev, 2011; this study) and the topical treatment by the spraying of felled trap trees (Kreutz, 2007; Kolev, 2011; Weber, 2011). Stem-injection of living trees can be a valuable tool, especially if single trees should be protected in recreational or urban areas. Two examples for the protection of single trees by stem-injection are the horse chestnut to prevent defoliation by *C. ohridella* and to prevent mortality of the green ash by larval feeding of *A. planipennis* in the bark. (Pavela and Bárnet, 2005; McKenzie et al., 2010). In the above mentioned cases, the stem-injection treatment proved to be a very effective method. In Norway spruce, establishment of breeding systems by adult beetles of *I. typographus* will interrupt the sap flow and kill the tree (Wermelinger et al., 2007). Therefore, Neem injected into the tree needs to prevent attack and oviposition of adult beetles in order to protect the tree. However, the use of Neem stem-injection into spruce trees failed to have such effects on attacking beetles (Kolev, 2011, this study).

As an alternative in forestry, stem-injection might be used to create a group of living trap trees in heavily infested stands in order to avoid development of offspring. In this case, beetles should be attracted by pheromone dispensers to these injected trees. However, this study showed that stem-injection with Neem into spruce does not reduce fecundity and fertility of adult *I. typographus* beetles, which will still be able to establish sister broods after re-emergence from the treated trees. Since first established sister broods contribute significantly to growth of the *I. typographus* population (Kritsch, 2005; Baier et al., 2007), additional injection of trees to trap re-emerging parental beetles is necessary. As a result, stem-injection of spruce for bark beetle control is time consuming and costly, and is not recommended as a sufficient control method in forestry.

As mentioned before, Neem application on felled trap trees proved to be highly effective at inhibiting the development of *I. typographus* progeny (Weber, 2011). Felled trap trees are used to control the beetle population in forest areas with locally high densities of infestations and this method combined with removal of damaged and infested trees is still the only reliable method to control *I. typographus* outbreaks (Wermelinger, 2004). In Austria, trap trees are either debarked, sprinkled, removed or treated topically with pyrethroid contact insecticides, which are currently the only insecticides allowed for use in forestry (Krehan et al., 2006) (BFW, 2011). However, unlike Neem, pyrethroids are potentially toxic to water organisms and vertebrates (Schröter and Weigersdorfer, 2007; Morgan, 2009) and therefore forbidden to be used close to water bodies, water reserves or natural conservation sites. In those areas, topically sprayed Neem could be an effective alternative.

Azadirachtin, the active ingredient of Neem, is quickly degraded under ambient conditions (Dureja and Johnson, 2000; Caboni et al., 2006). This restricts the efficacy of its use to a limited period of time; however, this fast detoxification on the other hand is a desirable effect in ecologically protected areas. My preliminary data gives no indication of adverse effects of Neem on the spectrum of natural enemies. Further studies about the effects on natural enemies of *I. typographus* after topical application on logs are recommended.

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6. Appendix

6.1. Breeding parameters

Tab.7: Density of breeding systems per bark area (BS /dm²) on segments in (Fig.14 a). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09).

BS density (n/dm²)	Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber	11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
12m	1,0	2,0	1,6	1,2	1,5	1,9	2,2	2,5	1,4	1,8	2,3	2,1	1,6
9m	1,6	1,4	1,6	1,9	1,4	1,8	2,0	2,7	2,0	2,1	1,7	1,8	1,3
6m	0,3	1,7	2,1	1,7	2,5	0,9	1,8	2,3	1,3	1,8	1,7	1,7	0,1
3m	0,0	1,7	1,1	1,5	2,0	0,0	2,0	1,3	0,4	1,2	1,7	0,0	0,4

Tab.8: Density of mother galleries per bark area (MG /dm²) on segments in (Fig.14 b). Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09).

MG density (n/dm²)	Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber	11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
12m	1,6	2,7	2,3	1,6	2,6	2,7	2,9	4,0	2,1	3,1	3,7	3,0	2,1
9m	1,0	2,1	2,5	2,8	1,9	3,0	3,1	3,9	3,1	3,7	2,7	3,0	2,0
6m	0,3	2,2	2,9	2,6	3,6	1,5	2,5	3,4	2,2	2,8	2,8	2,4	0,1
3m	0,0	2,5	1,4	2,1	3,2	0,0	3,4	1,9	1,1	2,1	2,1	0,0	0,8

Tab.9: Length of mother galleries (MG length cm) at different tree heights and treatments ($\dot{x}$, SD, n) in Fig.16. Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09).Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Segments marked with "-" had n≤3 breeding systems and could not be used for statistical analysis. Significant differences between tree heights of the same tree are marked with different capital letters (A, B, AB) (P<0.05).

MG length (cm)		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
	$\dot{x}$	5,5	5,7	7,5	5,5	9,8	6,0		6,1	7,1	8,8	7,3	7,8	7,4
12m	SD	2,2	1,8	1,7	0,7	2,9			0,5	1,6	2,3	3,5	1,8	1,3
	n	3	7	3	4	9	1	n/a	4	9	6	11	8	13
			B				-	-						
	$\dot{x}$		6,3	7,6	6,0	8,5		10,0	8,6	8,6	8,8	6,2	7,0	6,6
9m	SD		0,8	3,2	0,8	2,3		1,4	2,7	2,9	2,3	2,0	2,7	1,2
	n	n/a	3	7	5	10	n/a	2	9	9	3	12	12	7
		-	**AB**				-	-						
	$\dot{x}$	5,0	7,4	7,2		7,1	7,3	8,3	5,5	8,6	9,2	6,7	8,0	6,5
6m	SD		1,4	2,3		1,5	2,8	1,6	1,2	3,7	3,6	1,4	2,6	4,9
	n	1	9	10	n/a	7	9	8	8	7	9	12	10	2
		-	**AB**		-			**B**						-
	$\dot{x}$		8,4	7,9	10,5	10,6		6,2	6,9	7,8	8,1	6,7		7,1
3m	SD		1,8	2,1	1,4	2,8		2,3	2,0	2,5	2,8	1,7		1,5
	n	0	9	10	2	8	0	9	9	6	17	10	0	7
		-	**A**		-		-	**A**					-	

Tab.10: Proportion of mother gallery beyond last established larval gallery in percent of total mother gallery length (MG length beyond last LG) at different tree heights and treatments ($\dot{x}$, SD, n) in Fig.17. Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09).Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Segments marked with "-" had n≤3 breeding systems that were possible to analyse and could not be used for statistical analysis. Significant differences between the various trees at the same height are marked with different lower case letters (a, b, ab) (P<0.05).

MG after last LG (%)		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
	$\dot{x}$	29,9	48,2	37,6	38,5	29,1	41,7		30,7	30,5	38,4	38,0	34,4	58,6
12m	SD	9,8	18,4	14,7	6,4	9,9			7,8	13,5	6,6	21,8	6,9	16,1
	n	3	7	3	4	9	1	n/a	4	9	6	8	8	11
		ab	**ab**	**ab**	**ab**	**a**	**-**	**-**	**ab**	**a**	**ab**	**ab**	**a**	**b**
	$\dot{x}$		29,2	28,1	36,8	30,4		33,3	28,6	33,1	31,0	39,5	38,8	46,6
9m	SD		11,9	13,0	7,3	9,6			8,4	11,5	27,3	15,8	9,8	18,9
	n	n/a	3	7	5	10	n/a	1	9	9	3	9	9	7
		-					**-**	**-**						
	$\dot{x}$	10,0	38,7	38,9		33,4	40,2	29,9	37,4	33,4	25,7	31,7	29,7	
6m	SD		14,7	14,7		13,3	9,9	13,1	11,9	12,1	12,0	16,8	10,9	
	n	1	9	9	n/a	7	8	8	8	7	8	7	10	n/a
		-			**-**									**-**
	$\dot{x}$		30,9	28,8	14,9	20,5		23,4	28,0	37,1	31,8	46,4		41,1
3m	SD		7,2	13,3	8,7	11,5		10,8	10,3	16,4	21,0	26,9		15,1
	n	0	9	10	2	8	0	9	9	6	13	10	0	7
		-			**-**		**-**						**-**	

Tab.11: Egg density per cm mother gallery (Egg density/cm MG) at different tree heights and treatments ($\dot{x}$, SD, n) in Fig.18. Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Segments marked with "-" had n≤3 breeding systems that were possible to analyse and could not be used for statistical analysis. Significant differences between tree heights of the same tree are marked with different capital letters (A, B, AB) (P<0.05).

Egg density (n/ cm MG)		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
	$\dot{x}$	7,8	4,7	4,3	5,7	4,8	4,5		5,3	4,9	4,4	4,0	5,3	3,7
12m	SD	1,4	1,7	1,7	1,7	1,2			1,9	1,8	0,9	2,6	1,3	1,4
	n	3	7	3	4	9	1	n/a	4	9	6	11	8	13
							-	**-**						
	$\dot{x}$		4,7	5,4	5,0	5,3		4,6	5,2	4,2	5,4	5,2	4,7	4,1
9m	SD		1,4	1,1	0,7	1,4		0,1	0,6	1,1	0,7	1,6	0,7	1,2
	n	n/a	3	7	5	10	n/a	2	9	9	3	12	12	7
		-					**-**	**-**						
	$\dot{x}$	7,8	4,7	4,9		4,5	4,2	4,4	4,9	4,5	4,2	4,6	4,8	8,2
6m	SD		0,9	1,4		0,9	1,2	1,2	1,5	0,7	1,3	1,7	0,8	5,4
	n	1	9	10	n/a	7	9	8	8	7	9	12	10	2
		-			**-**			**B**						**-**
	$\dot{x}$		4,3	4,9	5,8	4,5		5,5	5,3	5,4	4,3	3,8		4,6
3m	SD		0,9	0,8	2,3	1,5		0,8	1,3	2,5	1,6	1,0		2,0
	n	0	9	10	2	8	0	9	9	6	17	10	0	7
		-			**-**		**-**	**A**					**-**	

Tab.12: Egg mortality in percent (%) at different tree heights and treatments (ẋ, SD, n) in Fig.19. Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Segments marked with "-" had n≤3 breeding systems that were possible to analyse and could not be used for statistical analysis. Significant differences between the various trees at the same height are marked with different lower case letters (a, b, ab) (P<0.05).

Egg mortality (%)		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
		15,9	11,0	3,8	8,3	13,5	3,7		13,7	19,8	16,3	72,7	33,9	29,5
12m	SD	7,2	4,2	3,8	7,3	11,7			14,2	12,4	18,5	24,9	27,1	26,3
	n	3	7	3	4	9	1	n/a	4	9	6	11	8	13
		a	**a**	**a**	**a**	**a**	**-**	**-**	**a**	**a**	**a**	**b**	**ab**	**ab**
	ẋ		18,5	15,9	12,4	17,0		6,4	5,2	20,2	16,2	64,3	46,0	58,1
9m	SD		21,7	6,1	12,6	11,7		2,3	5,2	21,7	12,1	33,5	48,3	28,7
	n	n/a	3	7	5	10	n/a	2	9	9	3	12	12	7
		-	**ab**	**ab**	**a**	**ab**	**-**	**-**	**a**	**ab**	**ab**	**b**	**ab**	**ab**
	ẋ	0,0	14,1	31,4		16,2	24,6	24,6	5,9	40,1	44,4	72,2	33,4	30,7
6m	SD		22,4	17,7		14,7	14,3	11,8	6,7	22,3	34,4	30,0	24,8	8,0
	n	1	9	10	n/a	7	9	8	8	7	9	12	10	2
		-	**a**	**ab**	**-**	**a**	**a**	**a**	**a**	**ab**	**ab**	**b**	**ab**	**-**
	ẋ		28,8	27,7	19,1	30,1		23,5	11,1	47,3	43,4	43,2		54,2
3m	SD		21,0	16,6	23,1	10,1		8,9	5,3	21,8	22,6	30,7		19,7
	n	0	9	10	2	8	0	9	9	6	17	10	0	7
		-			**-**		**-**						**-**	

Tab.13: Larval mortality in percent (%) at different tree heights and treatments (ẋ, SD, n) in Fig.20. Trees are grouped according to treatment into untreated control trees (control), trees injected with solvent solution (blank: tree 20B injected in 2009, tree 21B injected in 2008), trees injected with Neem in 2008 (Neem 08) and trees injected with Neem in 2009 (Neem 09). Tree heights marked with "n/a" (not available) could not be analysed due to intensive maturation feeding of filial beetles. Segments marked with "-" had n≤3 breeding systems that were possible to analyse and could not be used for statistical analysis. Significant differences between the various trees at the same height are marked with different lower case letters (a, b, ab) (P<0.05).

Larval mortality (%)		Control		Blank		Neem 08						Neem 09		
Treehight/ Treenumber		11X	19X	20B	21B	22J	28J	29J	32J	40J	41J	46J	49J	50J
	ẋ		1,4			9,2				5,9		73,9	46,7	25,1
12m	SD		2,4			13,1				8,3		22,9	50,3	23,1
	n	n/a	3	n/a	n/a	2	n/a	n/a	n/a	2	n/a	7	3	8
		-	**a**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**b**	**ab**	**a**
	ẋ			0,0	5,7	6,1			0,0	0,0	44,4	73,7	26,6	32,7
9m	SD				6,0	7,4			0,0			32,3	28,5	40,6
	n	n/a	n/a	1	3	4	n/a	n/a	2	1	1	6	5	4
		-	**-**	**-**			**-**	**-**	**-**	**-**	**-**			
	ẋ	0,0	3,5	0,0			24,0	0,0	6,8	37,7	65,1	57,8	35,6	14,7
6m	SD		4,9	0,0			30,1		8,3	29,4	56,4	36,4	38,2	10,7
	n	1	2	4	n/a	n/a	4	1	3	4	3	6	5	2
		-	**-**	**-**		**-**		**-**						**-**
	ẋ		40,1	37,4		0,0		1,9	3,2	50,7	54,1	62,2		36,2
3m	SD		33,1	38,5				3,2	6,5	44,8	27,8	33,1		41,6
	n	0	7	6	n/a	1	0	3	4	3	10	7	0	5
		-			**-**	**-**	**-**						**-**	

6.2. Examples for Ovaries

6.2.1. Ovaries of female parental beetles

Fig.31: Ovary of female parental beetle from untreated control tree 11X. The Corpus luteum (yellow, between ovarioles and oviduct) is indicating that the female recently laid eggs.

Fig.33: Ovary of female beetle from blank treated tree 12B. The Corpus luteum (yellow, between ovarioles and oviduct) is indicating that the female recently laid eggs.

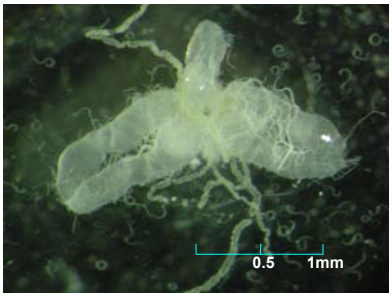

Fig.32: Ovary of beetle from Neem 2008 injected tree 32J. The Corpus luteum (yellow, between ovarioles and oviduct) is showing that eggs were recently laid

6.2.2. Ovaries of female F1-beetles

Ovaries of F1 beetles that emerged from trees felled on May 14:

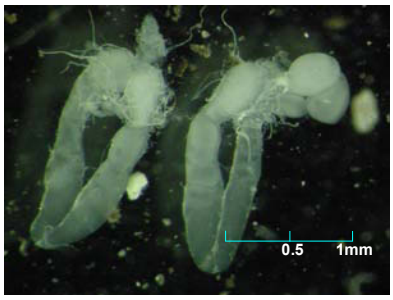

Fig.34: Ovary of female F1- beetle from the untreated control tree 11X.

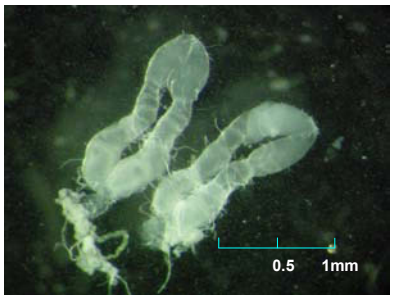

Fig.35: Ovary of female F1-beetle without corpus luteum from blank treated tree 12B.

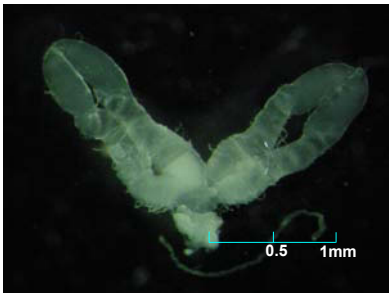

Fig.36: Mature ovary of female F1-beetle from Neem 2008 injected tree 32J.

Ovaries of F1 beetles that emerged from trees felled on June 22:

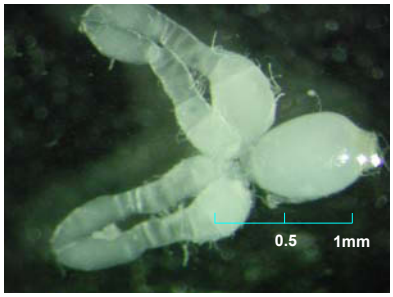

Fig.37: Ovary of female F1-beetle from untreated control tree 19X.

Fig.38: Ovary of female F1-beetles from Neem 2008 injected tree 29J.