
with a novel patented formulation of neem oil
...but
RP03
Dermanyssus gallinae (PRM) is the most harmful mite in laying hens
Happy for over 2 months!
Against the PRM
T1, day 0
T2, day 3
T3, day 6
block D (control)
kmax: 0.25 ± 0.05 log mite/day
Nres: 3.77± 0.02 log mite
block B (buffer)
kmax: 0.25 ± 0.03 log mite/day
Nres: 2.09 ± 0.05 log mite
block A (treated)
kmax: 0.36 ± 0.02 log mite/day
Nres: 0.75 ± 0.20 log mite
log D. gallinae
0 1 2 3 4 5 6
0 6 18 24 30 36 42 48 54 60 66 72 78 84 90 162
99% effective

Title**: Efficacy of a novel neem oil formulation (RP03^{TM}) to control the poultry red mite *Dermanyssus gallinae***

Running title: **Plant-derived product to control the poultry red mite**

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Abstract

Dermanyssus gallinae is the most harmful ectoparasite of laying hens, an occupational hazard for poultry workers, and an increasing threat to medical science *per se*. To control the mite there is an increasing demand for alternative products, including plant-derived acaricides. We investigated the efficacy of neem oil against *D. gallinae* on a heavily infested commercial laying egg farm. A novel formulation of 20% neem oil, diluted from a 2,400 ppm azadirachtin-concentrated stock (RP03^{TM}), was administered by nebulization three times in a week. Using corrugated cardboard traps, mite density was monitored before, during and after treatment and results were statistically analyzed. Mite populations in the treated block showed a 94.65%, 99.64% and 99.80% reduction after the first, second and third product administration, respectively. The reduction rate of the mite population was significantly higher for the treated block ($P<0.001$) compared to the control and buffer blocks. Results suggest strong bioactivity of neem, and specifically the patented neem-based RP03^{TM}, against *D. gallinae*. The treatment was most effective in the 10 days following the first application, and its effects persisted for over two months. Further studies will aim to overcome observed side effects of treatment caused by an oily layer on equipment and eggs.

Keywords: *Azadirachta indica*; *Dermanyssus gallinae*; acaricide; enriched colony system; laying hens; neem;,zoonosis.

Introduction

The poultry red mite *Dermanyssus gallinae* (De Geer 1778) is considered the most harmful ectoparasite of farmed poultry in Europe (**Sparagano *et al.*, 2014**). This haematophagous mite spends the day hidden in cracks and crevices of the chicken house, and feeds on the animals during the night (**Chauve, 1998**). In Europe *D. gallinae* is endemic, with infestation rates varying between countries. The most recent figures suggest that *D. gallinae* prevalence in laying hens varies from 20 to 90% in many EU countries, with an average prevalence of 83% (**Mul *et al.*, 2013**). Earlier estimates of percentage infestation in Italy were reported as 74% (**Cafiero *et al.*, 2008**), supporting increased significance of this pest over the last decade. *D. gallinae* is present in all poultry production systems: cages, aviaries and free range, both traditional and organic (**Hoglund *et al.*, 1995**). The impact of this pest, however, is most severe in laying hens (**Chauve, 1998**) due to the longer productive cycle in these systems when compared with broiler farms (**Giangaspero *et al.*, 2017**). Recent legislation banning conventional cage production (European Directive 1999/74/CE) has driven a shift towards more extensive and ‘enriched’ housing for laying hens in the EU. Such systems, however, tend to provide more complex environments that appear to favour *D. gallinae*, thus exacerbating the mites’ pest status. Reports of *D. gallinae* feeding upon mammals, including humans, are becoming increasingly common (**George *et al.*, 2015**) and it has been proposed as an occupational hazard for poultry workers (**Cafiero *et al.*, 2011**). Cases of human infestation are not limited to those working in close proximity to the mite, however, with increasing numbers of attacks also reported in private residences, hospitals, and office spaces, often due to synanthropic infested birds (**Cafiero *et al.*, 2009; George *et al.*, 2015**). Though most cases are quickly resolved and involve advantitious feeding only, an apparent rise in persistant human infestations in recent years should be cause for concern.

The main detrimental effect of *D. gallinae* infestation is stressing of hens, resulting in irritation, restlessness, feather pecking, and anemia in infested flocks. Heavy infestations have a negative impact on bird condition, growth rate, egg quality (through increased shell thinning and spotting) and production (**Chauve, 1998; Cosoroaba, 2001**).

Consequences of infestation are worsened due to the status of this species as a vector and reservoir for several bacterial and viral pathogens (**Valiente Moro *et al.* 2009; Camarda *et al.*, 2010, Circella *et al.*, 2011; Sparagano *et al.*, 2014)**.

Control of *D. gallinae* remains heavily reliant on the use of synthetic acaricides (i.e., carbaryl, organophosphates, permethrin). This is a matter of concern, however, as the continuous use of these products has already led to issues of resistance, treatment failure, presence of residues and animal and human welfare concerns (**Marangi *et al.*, 2009; Marangi *et al.*, 2012; Sparagano *et al.*, 2014**). Recognising the need to develop alternatives to conventional acaricides, the worldwide scientific community is investigating the efficacy of alternative control methods for *D. gallinae*, including both biopesticides and biological control. Several such products have now begun to penetrate the marketplace in some EU countries (e.g. spinosad), with a mounting body of evidence supporting strong future potential in plant-derived acaricides (**George *et al.*, 2014**).

Neem seed extract is proven to have activity against a wide range of pests of veterinary and medical significance, including *D. gallinae* (**Schmahl *et al.*, 2010**). Neem-based products contain compounds including azadirachtin and salanin that are known to be bioactive against mites and insects, whilst being relatively safe for other organisms (**Biswas *et al.*, 2002**). Azadirachtin acts by dispersing/blocking juvenile hormones in insects, interrupting growth and reproduction, also disrupting chitin synthesis in arachnids and insects. Salanin acts as a feeding deterrent in insects, with bioactivity also demonstrated for triterpenoids such as nimbin and nimbidin, which show antibacterial, antiviral and fungicidal properties (**George *et al.*, 2014**).

Although neem-based products have already been developed for use against *D. gallinae* and deployed either within traps (**Lundh *et al.*, 2005**) or as premise sprays (MiteStop® Falema, Switzerland), to date these have only been tested in poultry kept in free range and conventional cage systems, with only limited studies performed to support commercial benefit and a paucity of neem-based products available for potential use. Further research to develop a novel robust neem-based acaricide, and independently confirm efficacy of neem *per se* in a commercial setting, would thus be of benefit.

The above in mind, the aim of this study was to investigate the potential of a novel neem-based product RP03™ for the control of the poultry red mite *D. gallinae* under field conditions, in an enriched colony egg production system. RP03™ is a patented novel formulation (Farmaneem Srl) of an extract of the seeds of the neem tree (*Azadirachta indica*). The product is a spray formulation containing azadirachtin (0.24% min.), nimbin (0.4% min.), and salanin (0.6% min.).

Materials and methods

Site and animals

The study was carried out in an enriched cage unit on a commercial laying hen farm in the province of Brindisi (Apulia, Italy). The unit housed approximately 19,000 hens of a commercial genotype (Hy-line Brown and Hy-line White), which were approximately 14 months old at the start of the experiment and not previously housed in other cage facilities. The farm building was arranged in four blocks (A-D, **Fig. 1**) of cages, each consisting of two adjacent lines of cages, arranged over four tiers of 29 cages each (providing 116 cages per block and 464 cages in total), compliant with national and European regulation and welfare legislation. Twenty birds were housed in each cage. A forced ventilation system provided air circulation and negative pressure in the unit. Birds were fed *ad libitum* with a commercial layer mash and had continuous access to drinking water.

The farm was selected as the study site because of previous historical issues with *D. gallinae*, dating back several years. The infestation in the unit at the time of the study ranked at level IV according to the classification system of **Cox *et al.* (2009**), i.e., clusters of mites (groups of mites larger than 1 cm^2) were visible on the structures. In addition, preliminary inspections proved that the flock was properly managed and that no acaricide treatments had been applied in the 3 months prior to the trial commencing.

Study design

For assessing *D. gallinae* numbers, mites were collected in, and counted from, custom-made traps. Traps were prepared according to **Nordenfors *et al.* (1999)** with slight modifications. Namely, 100x140 mm pieces of corrugated cardboard were rolled and inserted into plastic tubes 10 cm long and with a diameter of 3 cm.

Traps were placed before, during and after the treatment which consisted of product application given three times during one week. Traps were left in situ for 48 hours at each sampling point prior to the third treatment, and for 72 hours at each sampling point thereafter. Collections for mite counts were performed at day 0 (before the first treatment) and 3, 6, 10, 18, 27, 34, 41, 50, 59, 69, 87 and 162 days after the first treatment. A detailed trapping and mite counting schedule is shown in **Supplementary Table 1.**

Mites were collected from cages on both sides of blocks A, B and D. Traps were placed in alternate cages, and between the selected cages, in order to cover a wider area and according to the routes tracked by mites to reach the hosts (**Fig. 1**). Forty traps per block (20 on each side) were placed, for a total of 120 traps per sampling occasion. At established times, the corrugated cardboard inserts in the traps were removed from the tubes and new inserts positioned ahead of subsequent samplings (**Supplementary Table 1**). Traps were processed for mite counting in 'blind' by the same individuals for consistency.

Once removed, each cardboard insert was placed individually in a plastic bag, taken to the laboratory and stored at -18 °C for 48 h to kill the mites present. After freezing, each trap was then opened and the mites were poured into a petri dish. Mites attached to the surfaces of the tubes were gently detached using a needle. Before counting, the mites were spread evenly in the petri dish and confirmed as *D. gallinae* according to the morphological keys by **Moss (1968)** and **Di Palma *et al.* (2012)**. All counts were made under a stereomicroscope (Leica, Wetzlar, Germany), though whenever more than 500 mites were present in a trap, their number was estimated by weighing. In these cases, the calibration standard was determined by weighing no less than five 100-mite aliquots.

Treatment application

The interconnected nature of cages within a block did not allow separation of each block into treatment replicates, so that treatment with the experimental neem formulation was administered to both lines of cages of Block A only. It should be pointed out that a dedicated experimental structure to serve as buffer zone (such as reported by **George *et al*., 2014**), could not be employed here due to the commercial nature of the facility. A formulation of 20% neem oil dilution, from a 2,400 ppm azadirachtin-concentrated stock (RP03™), was used and 150 L of this 20% solution was sprayed on the treated block by a pressurized hand-held lance sprayer (Spray Team SRL, Italy), with a particles size lower than 90-100 thousandths of a millimeter, covering all accessible surfaces of the cage walls and floors, also treating litter and animals present. Overall, a surface area of 457 m^2 was treated in Block A, equating to an overall volume of 237.42 m^3 of treated cage space. Approximately 0.32 L of neem solution was applied per m^2.

Block D was selected as the negative control, this being maximally spatially separated from the treated Block A, and was not subject to spraying. Block B was considered as a buffer block, in order to verify possible effects on mites due to the dispersion of RP03™. Block C was left untreated.

Records of hen mortality were kept during the study with *post-mortem* analysis undertaken on every dead bird.

Statistical analysis

In order to examine the effect of treatment on *D. gallinae* population response, the number of *D. gallinae* was preliminary standardized as $\log_{10}$ and analyzed to check for normality through the Shapiro-Wilk test. Then, log-values were used to build a variability plot, showing both raw data and median value *w* throughout time.

Then, a second standardization was run and the data reported as log decrease of *D. gallinae* against the starting population (log units at the beginning of the experiment – log units at time t). For this approach, each line of a block was treated as a separate sample and preliminarily analyzed through the Shapiro-Wilk test. On the log reduction values, a multifactorial ANOVA was run; time and position were use as categorical predictors. The predictor "time" had 12 different coded values (log after 3, 6, 10, 18, 27, 34, 41, 50, 59, 69, 87 and 162 days), whereas the predictor position had 6 coded values (A-line 1; A-line 2; B-line 1; B-line 2; D-line 1; D-line 2). The statistical treatment was performed using Statistica for

Windows, ver. 12.0 (Statsoft, Tulsa, Oklahoma). The analysis was corrected through a "dependence factor" estimated by the software. This factor takes into account that the two sides of each block could be not independent due to possible mite movement between them. The term time in the multifactorial ANOVA does not refer to a possible correlation time *vs* population (XY correlation); it is only a qualitative factor put in the analysis to elucidate that the population could be different for the treatment and the time of sampling. The multifactorial ANOVA was run as a GLM (general linear model) to assess the standard error of estimate of the whole model.

As a final step, the evolution of *D. gallinae* throughout time was fitted by using the Weibull/tail equation, as reported by **Geeraerd *et al.* (2005)**. This model allows the estimation of k_{max}, here akin to the rate of *D. gallinae* reduction, N_{res}.

Results

Pre-treatment infestation by D. gallinae

On day 0 (before treatment), mean counts of mites (± SD) were 48,284 ±15,864, 9,594 ±7,430, and 3,049 ± 4,689 in control, buffer and treated block, respectively (**Supplementary Table 2**).

Post-treatment D. gallinae *population monitoring evaluation*

According to the first step of the statistical approach, in the control block (**Fig. 2A)**, the initial median value was 4.65 log *D. gallinae*. This figure decreased to 3.25 log *D. gallinae* after 59 days and increased to 3.91 log *D. gallinae* at the end of the study period (162 days). In the buffer block (**Fig. 2B**), the initial median number was 3.90 log *D. gallinae* and was reduced to 1.56 log *D. gallinae* after 59 days, increasing to 2.77 log *D. gallinae* after 162 days. In the treated block (**Fig. 2C**), the mite population was reduced from 3.11 log *D. gallinae* to 0.39 log *D. gallinae* after 10 days, then experiencing a slight increase (up to 1.15 log units after 27 days), with a final decrease and a biostatic effect, as suggested by the median mite value, ranging from 0.48 to 0.98 log units.

The plots in **Fig. 2** show all raw data and suggest strong variability within each block. In addition, when both lines were used as replicates of a single block, the residuals of some samples did not follow a normal distribution; conversely, each line of a block, treated as a separate sample, showed a normal distribution and satisfied the basic assumptions of the analysis of variance (normal distribution of residuals, homoscedasticity). Therefore, the lines were treated as separate samples and a second standardization was done (log mite decrease) to compare the different blocks. Each sample was analysed as a function of the time and position (lines of each block).

Table 1 shows F-test outputs and the standardized effects. "Position" and "Time" were both significant as individual predictors, although the most significant was "position", according to the F-test. The log-reduction was also significantly affected by the interactive term position/time. ANOVA was run via a GLM (general linear model) and the standard error of estimate of the model was 0.53 log *D. gallinae*. In using a GLM the non-independence of the two sides of each block, and the time-dependency of the effect, could be taken into account in the analysis: however, the main goal of this research was to assess the effect of a main qualitative variable (treatment: control, buffer, treated row), a secondary qualitative variable (sides of each block) and a quantitative factor (time).

Time-dependence was expected, whereas the qualitative effect of the treatment (reduction or no reduction of mite population) could be better determined by a qualitative approach, like ANOVA.

In this respect, log-transformation and log reduction were used as a means to calculate a standard efficiency index that was independent from the initial mite count and less affected by the outliers.

A second output of a multifactorial ANOVA is the decomposition of the statistical hypothesis; as reported elsewhere (**Bevilacqua *et al.*, 2017**), the decomposition does not show actual values or effective trends, but a qualitative correlation on how each predictor acts on the dependent variable (log reduction of the number of *D. gallinae*). Concerning the effect of position (**Fig. 3A**), the highest mean reduction was found for Block A (2.1-2.3 log-reduction). In the buffer block (Block B), the two lines experienced a slight difference (1.5 log-reduction for the line 1 and 1.2 log-reduction for the line 2). Finally, in the control block (Block D), the mean reduction was 0.8 log-mite ($P<0.01$).

The effect of the predictor time (**Fig. 3B)** suggests that the population of *D. gallinae* experienced a decrease throughout time with the maximum reduction achieved after 59 days (P<0.01). **Fig. 3C** combines the predictor position and time and shows the log-reduction for each line in each block throughout time. In the treated block (A), the mean of mite-reduction was >90% after 3 days, then it increased to 99% or more. After 3 days, the mean log-reduction was 40-63% in the control and buffer blocks (D and B); thereafter, it increased and was >90% in the buffer block after 18 days (P<0.05).

An increase in log-reduction was also recovered in the control block (D), due to the main effect of the predictor time and to a decrease of mite population independently from the treatment. In this block, a mean effect of 90% (1-log reduction) was found after 41 days; moreover, the log-reduction for this block was always lower than the values found for the buffer and the treated blocks.

As indices of the effect of Neem on the mites, the log-reduction after the 1st, 2nd and 3rd treatment was evaluated: it was 94.65%, 99.64% and 99.80% in the treated block (Block A), 59.93%, 75.68% and 83.68% in the buffer (Block B) and 63.24%, 80.02% and 82.27% in the control (Block D).

Fig. 4 shows more intuitively the evolution of *D. gallinae* throughout time. As reported elsewhere, the mite population experienced a reduction throughout time in all the blocks; however, the rate of population decrease (0.36 log mite/day in the treated Block A *vs* 0.25 log mite/day in the control and buffer blocks, P at 0.023) and the residual population (0.75 log mite in the treated Block A, 2.09 log mite in Block B and 3.77 log mite in Block D) support a significant effect of the neem oil in controlling *D. gallinae* (where P=0.0001).

Hens' response to treatment

One hundred and seventy six birds, i.e., 0.9 % of the total number of hens present, died during the course of the study. This figure is below the normal mortality rate for Hy-line Brown and Hy-line White hens of the age used, which is 0.3-0.5% of the flock per month. Seven animals died prior to the application of treatment. *Post-mortem* examination performed on all birds showed no unusual causes of death. Chronic respiratory syndrome characterized by aerosacculits, catarrhal ovary and oviduct inflammation, caseous peritonitis, caused by *E. coli* and/or *Mycoplasma*, were the most frequently observed causes of death. Other deaths were due to accidental injuries. In no instance was any mortality event deemed treatment related.

Discussion

This study is the first to investigate neem efficacy in laying hens housed within an enriched colony system and supports that RP03^{TM} neem-based product is highly effective against *D. gallinae*. The product caused a very high reduction of the mite population, this exceeding 99% following the second treatment, and with long-lasting effects.

The results of mite trapping before the trial demonstrated that the *D. gallinae* population was not uniformly distributed across cage blocks. Differences in number of mites registered in one block compared to another were not completely unexpected, and they could be related to uncontrollable variables present in the laying system, such as location, humidity, air-flow, temperature, hen breed, etc. **(Nordenfors & Höglund, 2000; Arkle *et al*., 2004)**. Pre-existing differences in mite burden between control and treated blocks may be considered a limitation in the present study, as differences in the initial number of mites (i.e. a higher mite burden in the control block) could have potentially affected the output of statistical analyses. This event could not be avoided due to a number of factors, such as the limited availability of study sites and suitable facility design, intrinsic mite population variability within each facility, and inevitable lag times occurring between trap collection and assessment of trap contents. Because of the above, it was necessary to pre-set treatment block locations based on spatial arrangement alone and not on mite counts parameters (**Fig. 1**).

Nevertheless, to overcome this bias and avoid the effect of a possible intrinsic variability of each block, a preliminary standardization was done, by using the initial values as a baseline or internal reference for each control. This approach relies on the fact that an input factor (i.e. the use of neem oil in this study) affects the trend of the statistical population, but with the effect of the trend being independent from the initial value (**Bevilacqua *et al*., 2016**).

Treatment with neem-based product provided a thousand-fold reduction of the mite population after the second treatment (99.64%) in the current study, this reaching 99.80% after the third treatment. Even after the first treatment alone, a 94% reduction in the mite population in treated blocks was observed. In addition to this strong acaricidal effect and rapid knockdown of *D. gallinae*, the effect of treatment persisted for more than two months.

The reduction rate of the mite population was significantly higher for the treated block (P<0.001) compared to the buffer and control blocks. Nevertheless, it was also possible to observe a reduction in the population of the latter two blocks over the study duration. Though this could potentially be explained by the above mentioned fluctuations in environmental conditions, which are well known to affect *D. gallinae* population density (**Nordenfors & Höglund, 2000; Arkle *et al.*, 2004**), it is also possible that the dispersal of RP03™, due to the forced ventilation in the unit, contributed to reduce the number of mites in the blocks adjacent to the treated one, this being supported by the fact that the reduction seen was stronger nearer to the treated block. Trap position was the most significant variable, as well as the interactive term time/trap position. Trap position showed a mean mite log-reduction of ca. 2.2-2.4 for the treated block, while in the control and buffer areas the mean reduction was 0.8 and 1.3, respectively. These results were independent from the effect of time and suggest a strong bioactivity of neem.

After the first, the second and the third treatment, no side effects of neem were observed on laying hens, with no birds displaying anomalous behavior. Furthermore, anecdotal evidence provided by the poultry unit owner supported that no decrease in egg production was apparent post-treatment. Negative effects were, however, reported on the equipment (conveyor belt, and cage structures), on the floor and, more importantly, on eggs. The presence and the persistence of an oily film were observed for about 20 days after the third treatment, while a characteristic smell tainted the eggs laid in the 24 hours after treatment, likely due the contamination of the conveyor belt. Such side effects could be mitigated, at least partially, by using a reduced volume of solution, or by reducing the size of the aerosol droplets**.** Reduced repeat treatment schedules could also be of benefit in minimising negative effects. Due to the reclusive life cycle of *D. gallinae*, repeat application of up to three times in a week is often recommended (**Abel-Gaffar *et***

***al*., 2009; Locher *et al*., 2010**) to ensure that the generation emerging from hard-to-treat refugia post-initial treatment is targeted along with any existing nymphs and adults (**George *et al*., 2010).** However, given the high efficacy (>99%) of RP03™ after the second treatment, two treatments in a week might be considered as sufficient.

Worldwide, control of *D. gallinae* infestation is based almost exclusively on the use of synthetic acaricides. Despite more than 35 molecules having been tested for use against *D. gallinae* (including organophosphates, pyrethrins, pyrethroids, carbamates and amitraz), in practice, only a few products are licensed in the EU for use against this pest (**Sparagano *et al*., 2014)**. Perhaps as a consequence, several unlicensed or even banned (i.e. carbaryl) products are still widely used to fight infestations in some European countries (**Sparagano *et al*., 2014**). Recently, for example, mass recall of eggs across Europe and Asia occurred due to fipronil contamination, resulting in investigations into misuse/illegal use of this product by pest control to target *D. gallinae* (https://www.food.gov.uk/news-updates/news/2017/16463/update-on-fipronil-in-eggs), which involved also Italy (http://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?lingua=italiano&menu=notizie&p=dalministero&id=3058). To promote improved product use, there is an urgent need to identify alternative, cost-effective and efficacious control strategies. Among the natural compounds of use to this end (**Sparagano *et al*., 2014; George *et al*., 2014),** *in vivo* experiments using neem-impregnated cardboard traps have been shown to reduce *D. gallinae* populations by more than 90% **(Lundh *et al*., 2005)** and a neem registered product (MiteStop®), diluted at 1:33 with tap water, not only killed all stages of *D. gallinae*, but also did so more effectively than the synthetic organophosphate phoxim (**Abdel-Gaffar *et al*., 2009**). Given that prolonged efficacy was registered at 162 days post-treatment in the current study (up to 90% in the treated block), RP03™ appears to deliver significant residual control of *D. gallinae* (i.e. of at least 3 months).

Conclusion

This field study demonstrated a very high and long-lasting efficacy of neem-based product (RP03™)

against *D. gallinae* in enriched colony cages. For its characteristics of safety for animals and humans (**Biswas *et al.*, 2002**), azadirachtin-based products, and in particular the patented RP03^{TM}-product tested here, can be suggested for *D. gallinae* control, not only in the poultry sector, but also in private and public settings (residences, hospital, offices). Nevertheless, further studies should be undertaken to reduce the treatment schedule, optimise the neem oil concentration and consistency and independently confirm product safety. Such research should help to guarantee a high efficacy, high safety and long-lasting neem acaricide, overcoming potentially undesirable effects of the registered product on poultry equipment and eggs.

Ethical statement

The experiment described was authorized by the Ethical Committee for Animal Welfare of the University of Foggia (Prot. n. 004-2016). The treatments did not cause detriment to the birds, and no animals were sacrificed. The health and welfare conditions of the flock were assessed by independent expert veterinary personnel to ensure that animals did not receive any kind of damage of suffering during and after this study.

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The authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Fig. 1. Schematic representation of the experimental design used to test *in vivo* acaricidal activity of neem-based RP03^{TM} against *Dermanyssus gallinae*. The farm building was arranged in four blocks (A-D) of cages, each consisting of two adjacent lines of cages arranged over four tiers of 29 cages each (providing 116 cages per block and 464 cages in total). Traps were placed in an alternating pattern on each tier and each line.

Fig. 2. Variability plot for the population of *Dermanyssus gallinae* throughout time in the control (block D) (A), buffer (block B) (B) and treated (block A) (C). The points indicate the log value for each trap, the line shows the median value of each block.

Fig. 3. Decomposition of the statistical hypothesis for the predictors on the multifactorial ANOVA. A) Effect of the position; B) Effect of time; C) Effect of the interaction position/time. The bars indicate the 95%-confidence intervals.

Fig. 4. Evolution of *Dermanyssus gallinae*. k_{max} = rate of population decrease; $N_{res,}/$ = survivors (mean values ± standard error). T1 = 1st treatment; T2, 2nd treatment; T3, 3rd treatment.
The population evolution is fitted up to 87 days, though the last point shown indicates the mean values of the mite population after 162 days.

Supporting Information files

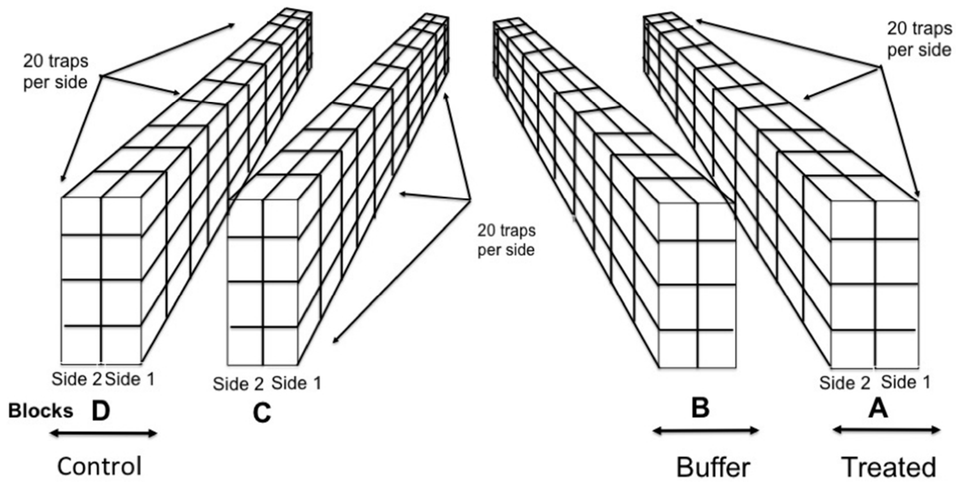
Table S1. Scheme of the trial schedule

Table S2. Number of *Dermanyssus gallinae* registered throughout the trial in Treated (A), Buffer (B) and Control (D) blocks, on one side of the block line (1), on the other side of the block line (2) and average on both lines (mean value of 1 and 2).

Table 1. Standardized effects of the multifactorial ANOVA. The analysis was run by using the GLM option in Statistica; the standard error of the model was 0.53 log *Dermanyssus gallinae*.

	SS	df	MS	F	P value
Intercept	3,262.845	1	3,262.845	11,590.47	<0.01
Position	461.976	5	92.395	328.21	<0.01
Time	161.962	11	14.724	52.30	<0.01
Position/time	67.919	55	1.235	4.39	<0.05
Error	385.107	1.368	0.282		

SS, sum of squares; MS, mean sum of squares; df, degree of freedom; F, Fisher test.



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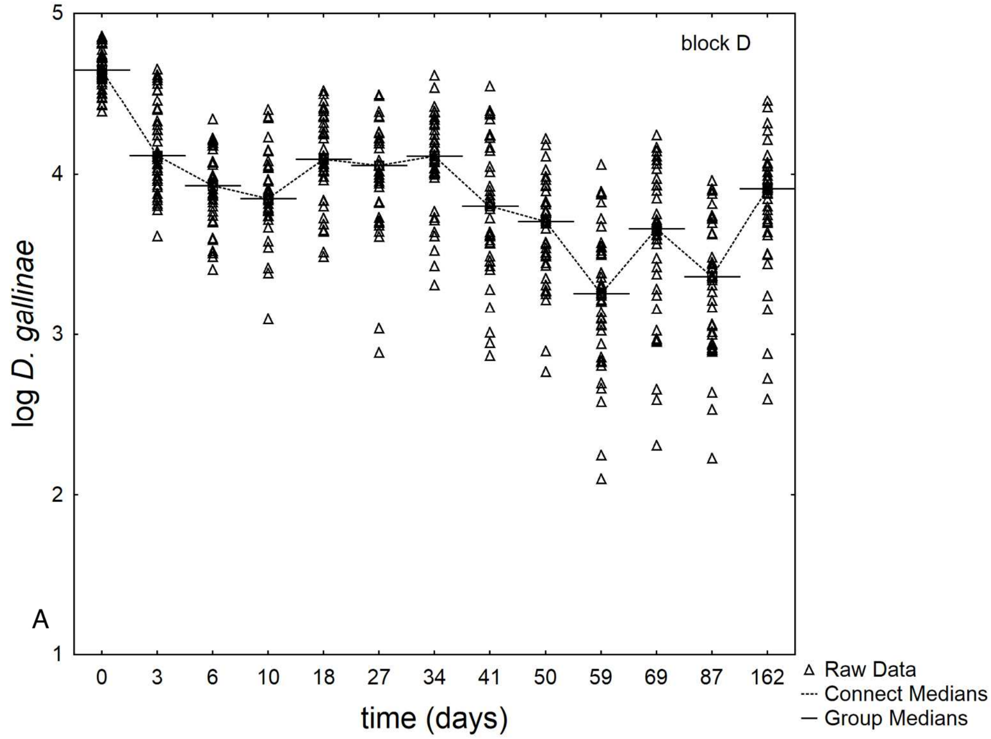
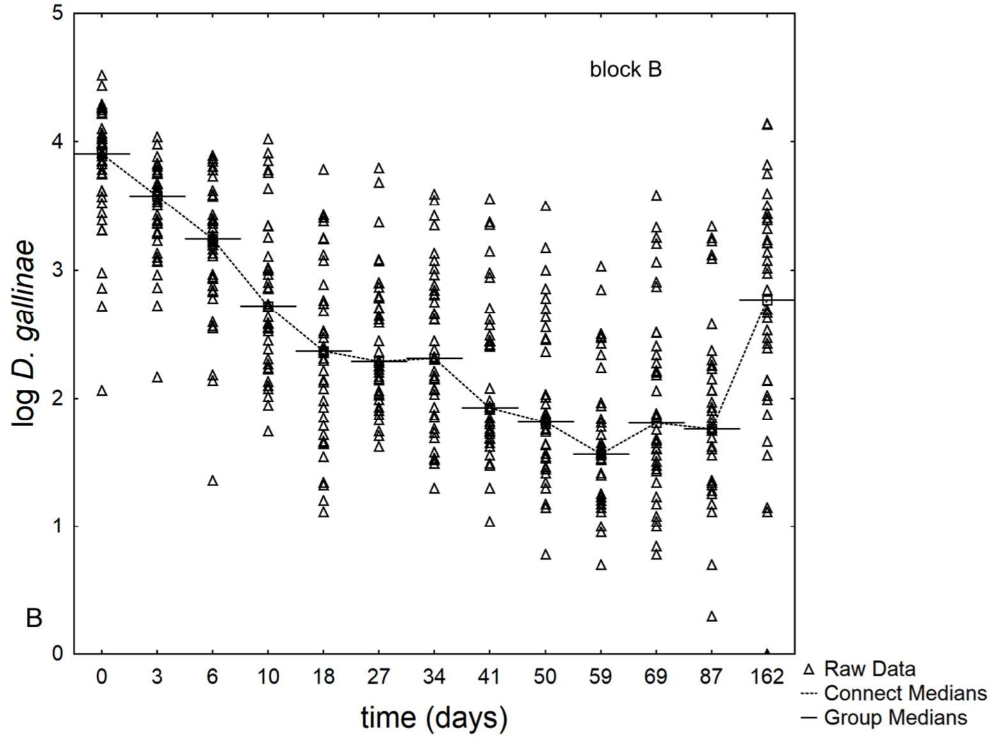


Fig. 2. Variability plot for the population of Dermanyssus gallinae throughout time in the control (block D) (A), buffer (block B) (B) and treated (block A) (C). The points indicate the log value for each trap, the line shows the median value of each block.

515x387mm (96 x 96 DPI)



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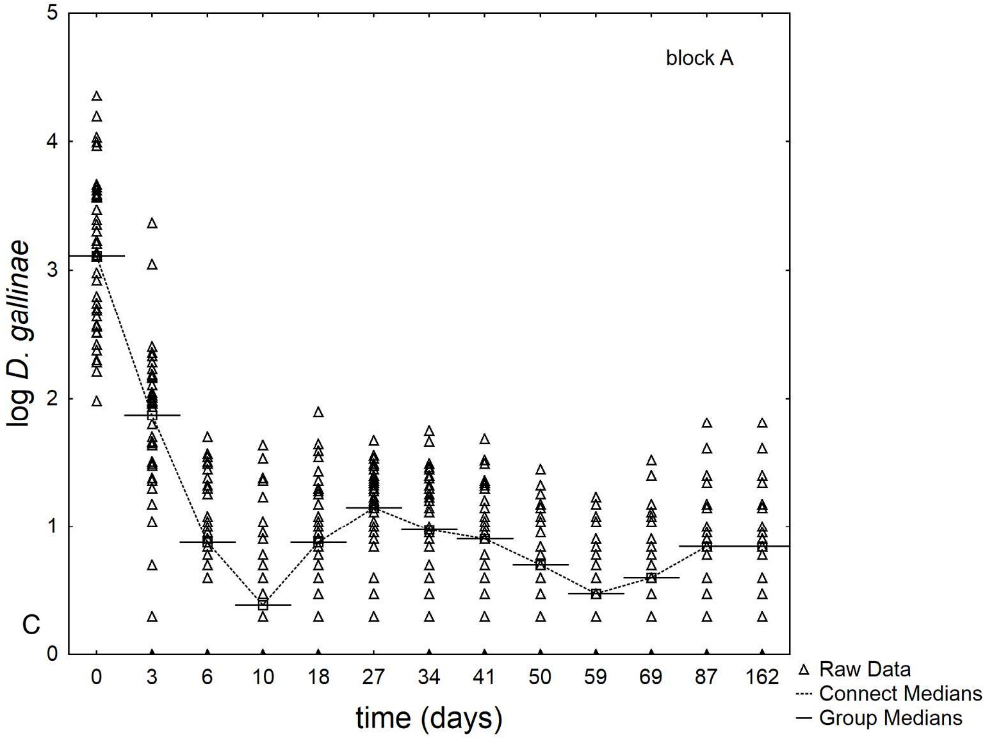

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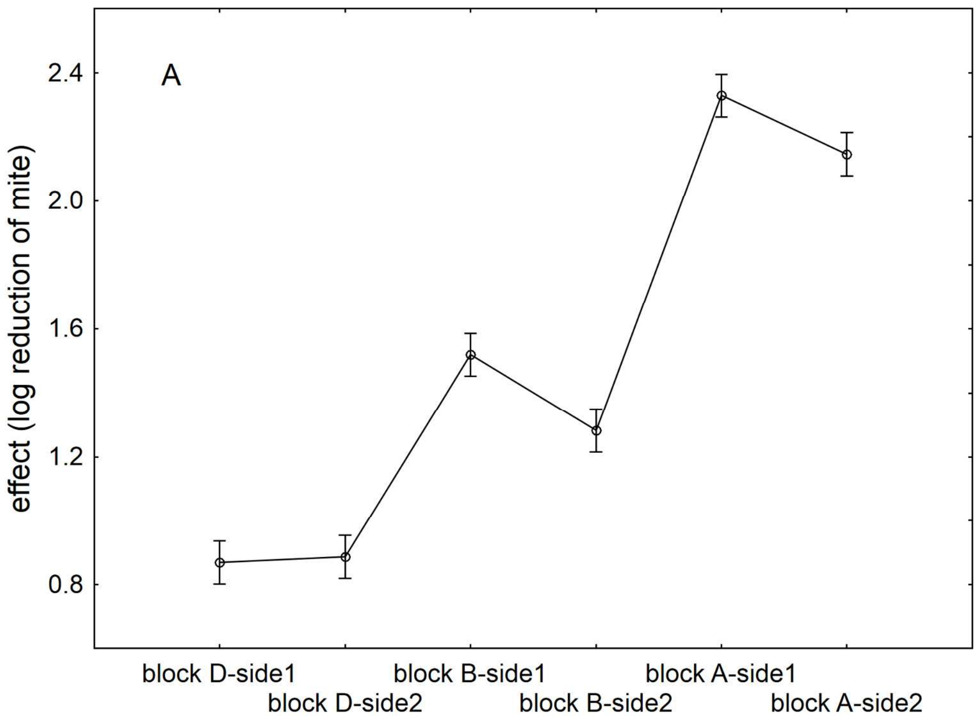
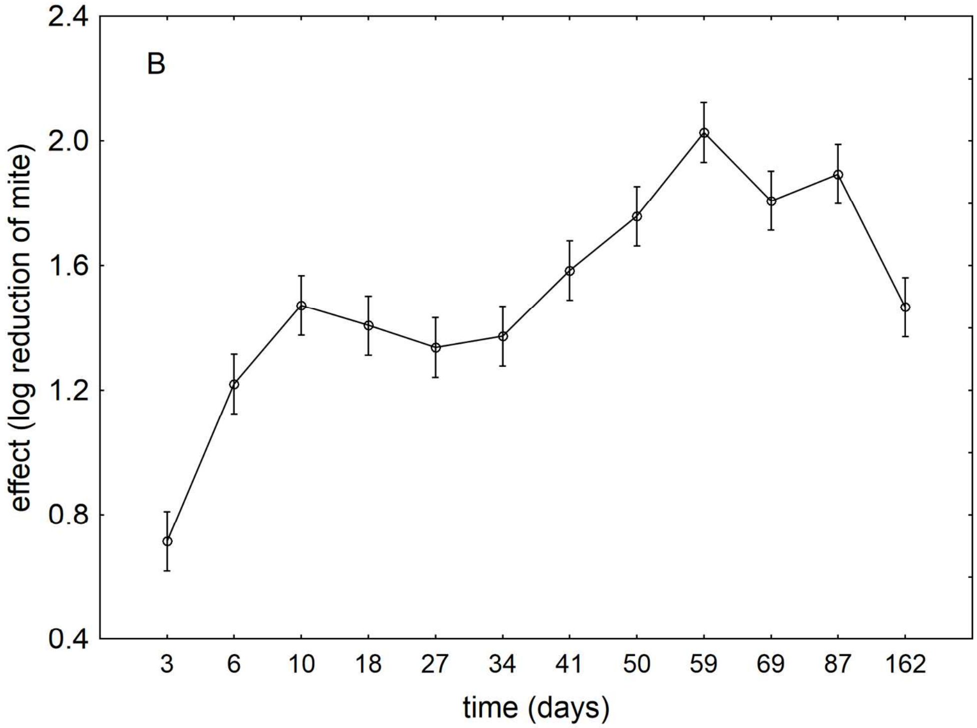


Fig. 3. Decomposition of the statistical hypothesis for the predictors on the multifactorial ANOVA. A) Effect of the position; B) Effect of time; C) Effect of the interaction position/time. The bars indicate the 95%-confidence intervals.

515x387mm (96 x 96 DPI)



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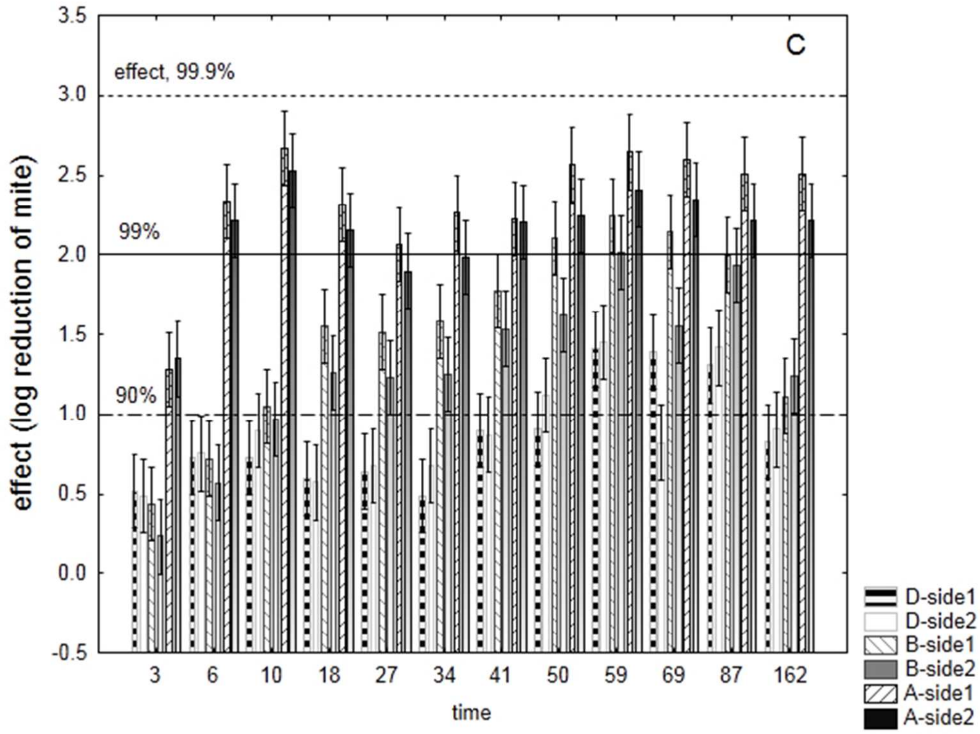

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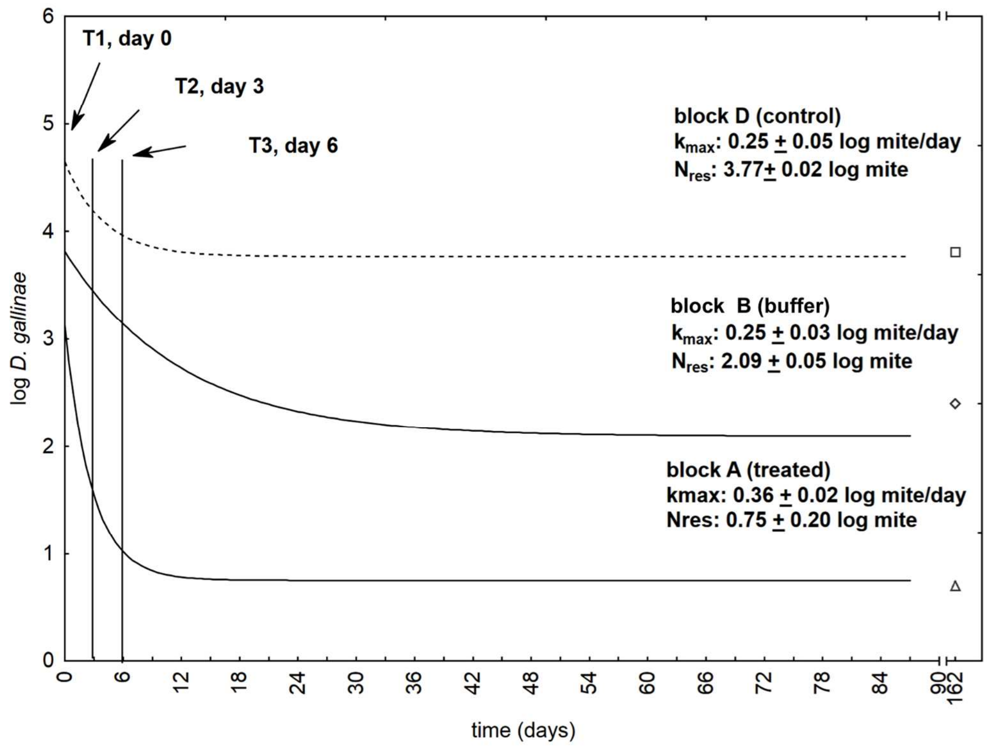

Fig. 4. Evolution of *Dermanyssus gallinae*. kmax = rate of population decrease; Nres,/ = survivors (mean values ± standard error). T1 = 1st treatment; T2, 2nd treatment; T3, 3rd treatment.‼ ┼ The population evolution is fitted up to 87 days, though the last point shown indicates the mean values of the mite population after 162 days. ‼ ┼

254x190mm (150 x 150 DPI)

Table S1. Scheme of the trial schedule

Day	Events
-3	Placement of traps
0 (T^1)	Cardboard removal and count **First treatment with RP03™** and Cardboard replacement
3 (T^2)	Cardboard removal and count, **Second treatment with RP03™** and Cardboard replacement
6 ($T^{3)}$	Cardboard removal and count **Third treatment with RP03™**
7	Cardboard replacement
10	Cardboard removal and count
15	Cardboard replacement
18	Cardboard removal and count
24	Cardboard replacement
27	Cardboard removal and count
31	Cardboard replacement
34	Cardboard removal and count
38	Cardboard replacement
41	Cardboard removal and count
47	Cardboard replacement
50	Cardboard removal and count
56	Cardboard replacement
59	Cardboard removal and count
66	Cardboard replacement
69	Cardboard removal and count
84	Cardboard replacement
87	Cardboard removal and count
159	Cardboard replacement
162	Cardboard removal and count

T^1: 1st treatment; T^2: 2nd treatment; $T^{3:}$ 3rd treatment

Table S2. Number of *Dermanyssus gallinae* registered throughout the trial in Treated (A), Buffer (B) and Control (D) blocks, on one side of the block line (1), on the other side of the block line (2) and average on both lines (mean value of 1 and 2)

	Mite mean count ± SD								
Days	Block D1	Block D2	Block D (1and 2)	Block B1	Block B2	Block B (1and 2)	Block A1	Block A2	Block A (1and 2)
-3 (Pre-treatment)	45,632 ± 16,518	50,935 ± 15,131	48,284 ± 15.864	11,275 ± 6,998	7,913 ± 7,641	9,594 ± 7,430	3,132 ± 3,814	2,965 ± 5,528	3,049± 4,689
3 (After the first treatment)	15,688 ± 10,121	19,809 ± 13,095	17,640 ± 11,651	3,889 ± 2,530	3,798 ± 2,469	3,844 ± 2,468	152 ± 237	175 ± 509	163 ± 392
6 (After the second treatment)	9,491 ± 4,884	9,802 ± 5,076	9,655 ± 4,921	2,701 ± 2,441	1,965 ± 1,737	2,333 ± 2,124	15 ± 15	8 ± 6	11 ± 12
10 After the third treatment	10,062 ± 6,823	7,064 ± 3,190	8,684 ± 5,602	2,143 ± 3,187	989 ± 1,363	1,566 ± 2,489	8 ± 12	4 ± 5	6 ± 10
18	13,363 ± 8,235	15,412 ± 8,315	14,388 ± 8,234	572 ± 831	1,102 ± 1,842	837 ± 1,436	13 ± 12	12 ± 18	12 ± 15
27	12,344 ± 7,093	12,992 ± 8,470	12,668 ± 7,718	384 ± 360	901 ± 1,694	642 ± 1,237	16 ± 9	16 ± 12	16 ± 10
34	16,765 ± 9,842	12,400 ± 6,464	14,582 ± 8,511	513 ± 799	727 ± 1,070	618 ± 935	13 ± 13	14 ± 11	14 ± 12
41	7,810 ± 6,576	10,311 ± 9,640	9,061 ± 8,243	232 ± 305	585 ± 1,001	409 ± 752	14 ± 11	9 ± 8	11 ± 11
50	6,465 ± 3,759	4,817 ± 3,646	5,641 ± 3,749	113 ± 139	419 ± 760	266 ± 561	6 ± 5	8 ± 7	7 ± 6
59	2,579 ± 2,833	2,707 ± 2,256	2,643 ± 2,529	75 ± 88	160 ± 277	118 ± 207	5 ± 4	5 ± 4	5 ± 4
69	2,752 ± 2,596	8,354 ± 4,229	5,553 ± 4,477	163 ± 353	580 ± 990	372 ± 763	6 ± 7	7 ± 6	6 ± 7
87	3,189 ± 2,720	2,586 ± 2,047	2,888 ± 2,395	281 ± 496	261 ± 601	271 ± 544	17 ± 53	6 ± 9	11 ± 38
162	9,913 ± 8,020	7,410 ± 3,837	8,662 ± 6,334	2,158 ± 3,167	1,738 ± 3,322	1,948 ± 3,209	9 ± 14	10 ± 10	9 ± 12

Neem Oil
(RP03™)

HIGHLIGHTS

- Control of *Dermanyssus gallinae*, the poultry red mite, relies heavily on the use of chemicals
- There is an urgent need to develop alternative products to avoid resistance and residues
- A novel formulation of neem oil to treat laying hens against *D. gallinae* has been tested
- The mite population was reduced by 99% after the second treatment, and effects persisted over 2 months
- This is the first study on neem efficacy in laying hens housed within an enriched colony


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No anymore chemicals! A novel formulation of neem oil reduce the mite poupulation by 99% after the second treatment.