Medical and Veterinary Entomology

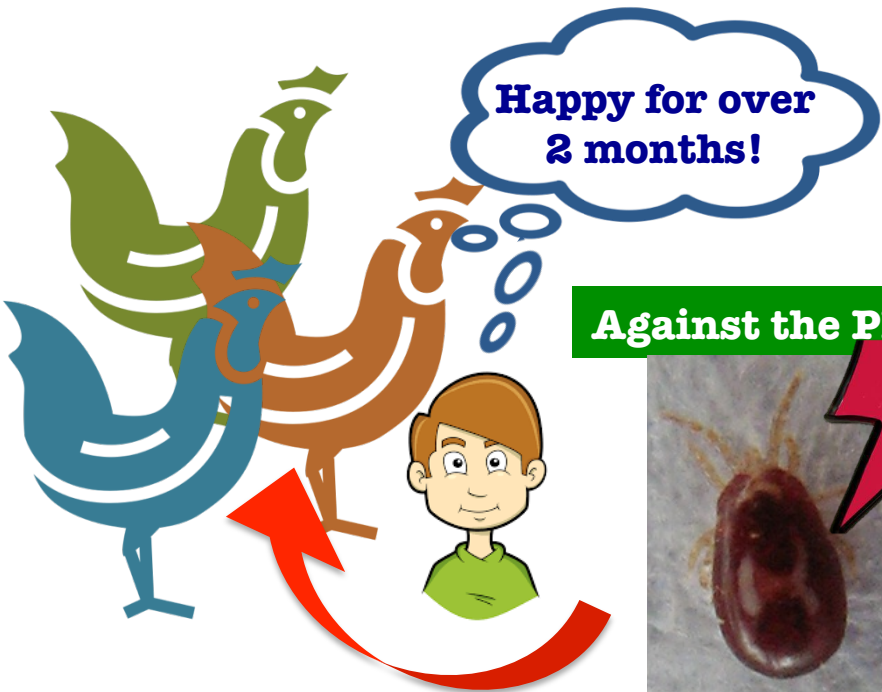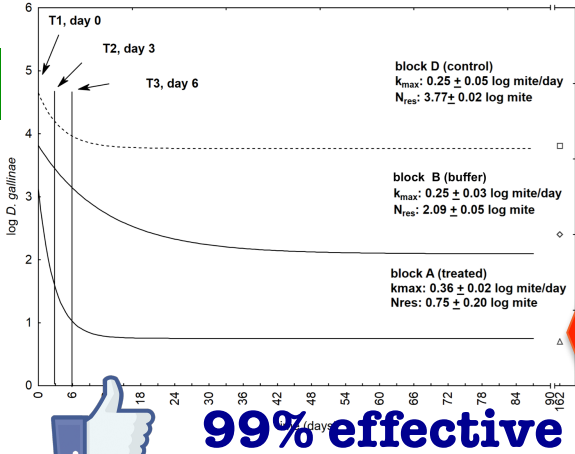**with a novel patented formulation of neem oil**

...but

Dermanyssus gallinae (PRM) is the most harmful mite in laying hens

RP03

Happy for over 2 months!

Against the PRM

99% effective

T1, day 0
T2, day 3
T3, day 6

block D (control)
k_{max}: 0.25 $\pm$ 0.05 log mite/day
N_{res}: 3.77 $\pm$ 0.02 log mite

block B (buffer)
k_{max}: 0.25 $\pm$ 0.03 log mite/day
N_{res}: 2.09 $\pm$ 0.05 log mite

block A (treated)
kmax: 0.36 $\pm$ 0.02 log mite/day
Nres: 0.75 $\pm$ 0.20 log mite

log *D. gallinae*

1   Title**: Efficacy of a novel neem oil formulation (RP03[TM]) to control the poultry red mite**

2   *Dermanyssus gallinae*

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

4

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25 **Abstract**

26 *Dermanyssus gallinae* is the most harmful ectoparasite of laying hens, an occupational hazard for poultry

27 workers, and an increasing threat to medical science *per se*. To control the mite there is an increasing

28 demand for alternative products, including plant-derived acaricides.   We investigated the efficacy of

29 neem oil against *D. gallinae* on a heavily infested commercial laying egg farm. A novel formulation of

30 20% neem oil, diluted from a 2,400 ppm azadirachtin-concentrated stock (RP03^{TM}), was administered by

31 nebulization three times in a week. Using corrugated cardboard traps, mite density was monitored before,

32 during and after treatment and results were statistically analyzed. Mite populations in the treated block

33 showed a 94.65%, 99.64% and 99.80% reduction after the first, second and third product administration,

34 respectively. The reduction rate of the mite population was significantly higher for the treated block

35 ($P<0.001$) compared to the control and buffer blocks. Results suggest strong bioactivity of neem, and

36 specifically the patented neem-based RP03^{TM}, against *D. gallinae*. The treatment was most effective in

37 the 10 days following the first application, and its effects persisted for over two months. Further studies

38 will aim to overcome observed side effects of treatment caused by an oily layer on equipment and eggs.

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Introduction

46 The poultry red mite *Dermanyssus gallinae* (De Geer 1778) is considered the most harmful ectoparasite

47 of farmed poultry in Europe (**Sparagano *et al.*, 2014**). This haematophagous mite spends the day hidden

48 in cracks and crevices of the chicken house, and feeds on the animals during the night (**Chauve, 1998**). In

49 Europe *D. gallinae* is endemic, with infestation rates varying between countries. The most recent figures

50 suggest that *D. gallinae* prevalence in laying hens varies from 20 to 90% in many EU countries, with an

51 average prevalence of 83% (**Mul *et al.*, 2013**). Earlier estimates of percentage infestation in Italy were

52 reported as 74% (**Cafiero *et al.*, 2008**), supporting increased significance of this pest over the last decade.

53 *D. gallinae* is present in all poultry production systems: cages, aviaries and free range, both traditional

54 and organic (**Hoglund *et al.*, 1995**). The impact of this pest, however, is most severe in laying hens

55 (**Chauve, 1998**) due to the longer productive cycle in these systems when compared with broiler farms

56 (**Giangaspero *et al.*, 2017**). Recent legislation banning conventional cage production (European

57 Directive 1999/74/CE) has driven a shift towards more extensive and 'enriched' housing for laying hens

58 in the EU. Such systems, however, tend to provide more complex environments that appear to favour *D.*

59 *gallinae*, thus exacerbating the mites' pest status. Reports of *D. gallinae* feeding upon mammals,

60 including humans, are becoming increasingly common (**George *et al.*, 2015**) and it has been proposed as

61 an occupational hazard for poultry workers (**Cafiero *et al.*, 2011**). Cases of human infestation are not

62 limited to those working in close proximity to the mite, however, with increasing numbers of attacks also

63 reported in private residences, hospitals, and office spaces, often due to synanthropic infested birds

64 (**Cafiero *et al.*, 2009; George *et al.*, 2015**). Though most cases are quickly resolved and involve

65 advantitious feeding only, an apparent rise in persistant human infestations in recent years should be

66 cause for concern.

67 The main detrimental effect of *D. gallinae* infestation is stressing of hens, resulting in irritation,

68 restlessness, feather pecking, and anemia in infested flocks. Heavy infestations have a negative impact on

69 bird condition, growth rate, egg quality (through increased shell thinning and spotting) and production

70 (**Chauve, 1998; Cosoroaba, 2001**).

71   Consequences of infestation are worsened due to the status of this species as a vector and reservoir for

72   several bacterial and viral pathogens (**Valiente Moro *et al.* 2009; Camarda *et al.*, 2010, Circella *et al.*,**

73   **2011; Sparagano *et al.*, 2014)**.

74   Control of *D. gallinae* remains heavily reliant on the use of synthetic acaricides (i.e., carbaryl,

75   organophosphates, permethrin). This is a matter of concern, however, as the continuous use of these

76   products has already led to issues of resistance, treatment failure, presence of residues and animal and

77   human welfare concerns (**Marangi *et al.*, 2009; Marangi *et al.*, 2012; Sparagano *et al.*, 2014)**.

78   Recognising the need to develop alternatives to conventional acaricides, the worldwide scientific

79   community is investigating the efficacy of alternative control methods for *D. gallinae*, including both

80   biopesticides and biological control. Several such products have now begun to penetrate the marketplace

81   in some EU countries (e.g. spinosad), with a mounting body of evidence supporting strong future

82   potential in plant-derived acaricides (**George *et al.*, 2014**).

83   Neem seed extract is proven to have activity against a wide range of pests of veterinary and medical

84   significance, including *D. gallinae* (**Schmahl *et al.*, 2010**). Neem-based products contain compounds

85   including azadirachtin and salanin that are known to be bioactive against mites and insects, whilst being

86   relatively safe for other organisms (**Biswas *et al.*, 2002**). Azadirachtin acts by dispersing/blocking

87   juvenile hormones in insects, interrupting growth and reproduction, also disrupting chitin synthesis in

88   arachnids and insects. Salanin acts as a feeding deterrent in insects, with bioactivity also demonstrated for

89   triterpenoids such as nimbin and nimbidin, which show antibacterial, antiviral and fungicidal properties

90   (**George *et al.*, 2014**).

91   Although neem-based products have already been developed for use against *D. gallinae* and deployed

92   either within traps (**Lundh *et al.*, 2005**) or as premise sprays (MiteStop® Falema, Switzerland), to date

93   these have only been tested in poultry kept in free range and conventional cage systems, with only limited

94   studies performed to support commercial benefit and a paucity of neem-based products available for

95   potential use. Further research to develop a novel robust neem-based acaricide, and independently

96   confirm efficacy of neem *per se* in a commercial setting, would thus be of benefit.

97   The above in mind, the aim of this study was to investigate the potential of a novel neem-based product

98   RP03[TM] for the control of the poultry red mite *D. gallinae* under field conditions, in an enriched colony

99   egg production system. RP03[TM] is a patented novel formulation (Farmaneem Srl) of an extract of the

100   seeds of the neem tree (*Azadirachta indica*). The product is a spray formulation containing azadirachtin

101   (0.24% min.), nimbin (0.4% min.), and salanin (0.6% min.).

102

103   **Materials and methods**

104   *Site and animals*

105   The study was carried out in an enriched cage unit on a commercial laying hen farm in the province of

106   Brindisi (Apulia, Italy). The unit housed approximately 19,000 hens of a commercial genotype (Hy-line

107   Brown and Hy-line White), which were approximately 14 months old at the start of the experiment and

108   not previously housed in other cage facilities. The farm building was arranged in four blocks (A-D, **Fig.**

109   **1**) of cages, each consisting of two adjacent lines of cages, arranged over four tiers of 29 cages each

110   (providing 116 cages per block and 464 cages in total), compliant with national and European regulation

111   and welfare legislation. Twenty birds were housed in each cage. A forced ventilation system provided air

112   circulation and negative pressure in the unit. Birds were fed *ad libitum* with a commercial layer mash and

113   had continuous access to drinking water.

114   The farm was selected as the study site because of previous historical issues with *D. gallinae*, dating back

115   several years. The infestation in the unit at the time of the study ranked at level IV according to the

116   classification system of **Cox *et al.* (2009)**, i.e., clusters of mites (groups of mites larger than 1 cm^2) were

117   visible on the structures. In addition, preliminary inspections proved that the flock was properly managed

118   and that no acaricide treatments had been applied in the 3 months prior to the trial commencing.

119

120   *Study design*

121   For assessing *D. gallinae* numbers, mites were collected in, and counted from, custom-made traps. Traps

122   were prepared according to **Nordenfors *et al.* (1999)** with slight modifications. Namely, 100x140 mm

123   pieces of corrugated cardboard were rolled and inserted into plastic tubes 10 cm long and with a diameter

124   of 3 cm.

125   Traps were placed before, during and after the treatment which consisted of product application given

126   three times during one week. Traps were left in situ for 48 hours at each sampling point prior to the third

127   treatment, and for 72 hours at each sampling point thereafter. Collections for mite counts were performed

128   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

129   treatment. A detailed trapping and mite counting schedule is shown in **Supplementary Table 1.**

130   Mites were collected from cages on both sides of blocks A, B and D. Traps were placed in alternate

131   cages, and between the selected cages, in order to cover a wider area and according to the routes tracked

132   by mites to reach the hosts (**Fig. 1**). Forty traps per block (20 on each side) were placed, for a total of 120

133   traps per sampling occasion. At established times, the corrugated cardboard inserts in the traps were

134   removed from the tubes and new inserts positioned ahead of subsequent samplings (**Supplementary**

135   **Table 1**). Traps were processed for mite counting in 'blind' by the same individuals for consistency.

136   Once removed, each cardboard insert was placed individually in a plastic bag, taken to the laboratory and

137   stored at -18 °C for 48 h to kill the mites present. After freezing, each trap was then opened and the mites

138   were poured into a petri dish. Mites attached to the surfaces of the tubes were gently detached using a

139   needle. Before counting, the mites were spread evenly in the petri dish and confirmed as *D. gallinae*

140   according to the morphological keys by **Moss (1968)** and **Di Palma et al. (2012)**. All counts were made

141   under a stereomicroscope (Leica, Wetzlar, Germany), though whenever more than 500 mites were

142   present in a trap, their number was estimated by weighing. In these cases, the calibration standard was

143   determined by weighing no less than five 100-mite aliquots.

144

145   *Treatment application*

146   The interconnected nature of cages within a block did not allow separation of each block into treatment

147   replicates, so that treatment with the experimental neem formulation was administered to both lines of

148   cages of Block A only. It should be pointed out that a dedicated experimental structure to serve as buffer

149   zone (such as reported by **George *et al.*, 2014**), could not be employed here due to the commercial nature

150   of the facility. A formulation of 20% neem oil dilution, from a 2,400 ppm azadirachtin-concentrated

151   stock (RP03[TM]), was used and 150 L of this 20% solution was sprayed on the treated block by a

152   pressurized hand-held lance sprayer (Spray Team SRL, Italy), with a particles size lower than 90-100

153   thousandths of a millimeter, covering all accessible surfaces of the cage walls and floors, also treating

154   litter and animals present. Overall, a surface area of 457 m^2 was treated in Block A, equating to an

155   overall volume of 237.42 m^3 of treated cage space. Approximately 0.32 L of neem solution was applied

156   per m^2.

157   Block D was selected as the negative control, this being maximally spatially separated from the treated

158   Block A, and was not subject to spraying. Block B was considered as a buffer block, in order to verify

159   possible effects on mites due to the dispersion of RP03[TM]. Block C was left untreated.

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

161   bird.

162

163   *Statistical analysis*

164   In order to examine the effect of treatment on *D. gallinae* population response, the number of *D. gallinae*

165   was preliminary standardized as $\log_{10}$ and analyzed to check for normality through the Shapiro-Wilk test.

166   Then, log-values were used to build a variability plot, showing both raw data and median value *w*

167   throughout time.

168   Then, a second standardization was run and the data reported as log decrease of *D. gallinae* against the

169   starting population (log units at the beginning of the experiment – log units at time t). For this approach,

170   each line of a block was treated as a separate sample and preliminarily analyzed through the Shapiro-

171   Wilk test. On the log reduction values, a multifactorial ANOVA was run; time and position were use as

172   categorical predictors. The predictor "time" had 12 different coded values (log after 3, 6, 10, 18, 27, 34,

173   41, 50, 59, 69, 87 and 162 days), whereas the predictor position had 6 coded values (A-line 1; A-line 2;

174   B-line 1; B-line 2; D-line 1; D-line 2). The statistical treatment was performed using Statistica for

175   Windows, ver. 12.0 (Statsoft, Tulsa, Oklahoma). The analysis was corrected through a "dependence

176   factor" estimated by the software. This factor takes into account that the two sides of each block could be

177   not independent due to possible mite movement between them. The term time in the multifactorial

178   ANOVA does not refer to a possible correlation time *vs* population (XY correlation); it is only a

179   qualitative factor put in the analysis to elucidate that the population could be different for the treatment

180   and the time of sampling. The multifactorial ANOVA was run as a GLM (general linear model) to assess

181   the standard error of estimate of the whole model.

182   As a final step, the evolution of *D. gallinae* throughout time was fitted by using the Weibull/tail equation,

183   as reported by **Geeraerd *et al.* (2005)**. This model allows the estimation of k_{max}, here akin to the rate of

184   *D. gallinae* reduction, N_{res}.

185

186   **Results**

187   *Pre-treatment infestation by* D. gallinae

188   On day 0 (before treatment), mean counts of mites (± SD) were 48,284 ±15,864, 9,594 ±7,430, and 3,049

189   ± 4,689 in control, buffer and treated block, respectively (**Supplementary Table 2**).

190

191   *Post-treatment* D. gallinae *population monitoring evaluation*

192   According to the first step of the statistical approach, in the control block (**Fig. 2A)**, the initial median

193   value was 4.65 log *D. gallinae*. This figure decreased to 3.25 log *D. gallinae* after 59 days and increased

194   to 3.91 log *D. gallinae* at the end of the study period (162 days). In the buffer block (**Fig. 2B**), the initial

195   median number was 3.90 log *D. gallinae* and was reduced to 1.56 log *D. gallinae* after 59 days,

196   increasing to 2.77 log *D. gallinae* after 162 days. In the treated block (**Fig. 2C**), the mite population was

197   reduced from 3.11 log *D. gallinae* to 0.39 log *D. gallinae* after 10 days, then experiencing a slight

198   increase (up to 1.15 log units after 27 days), with a final decrease and a biostatic effect, as suggested by

199   the median mite value, ranging from 0.48 to 0.98 log units.

200   The plots in **Fig. 2** show all raw data and suggest strong variability within each block. In addition, when

201   both lines were used as replicates of a single block, the residuals of some samples did not follow a normal

202   distribution; conversely, each line of a block, treated as a separate sample, showed a normal distribution

203   and satisfied the basic assumptions of the analysis of variance (normal distribution of residuals,

204   homoscedasticity). Therefore, the lines were treated as separate samples and a second standardization was

205   done (log mite decrease) to compare the different blocks. Each sample was analysed as a function of the

206   time and position (lines of each block).

207   **Table 1** shows F-test outputs and the standardized effects. "Position" and "Time" were both significant

208   as individual predictors, although the most significant was "position", according to the F-test. The log-

209   reduction was also significantly affected by the interactive term position/time. ANOVA was run via a

210   GLM (general linear model) and the standard error of estimate of the model was 0.53 log *D. gallinae*. In

211   using a GLM the non-independence of the two sides of each block, and the time-dependency of the

212   effect, could be taken into account in the analysis: however, the main goal of this research was to assess

213   the effect of a main qualitative variable (treatment: control, buffer, treated row), a secondary qualitative

214   variable (sides of each block) and a quantitative factor (time).

215   Time-dependence was expected, whereas the qualitative effect of the treatment (reduction or no reduction

216   of mite population) could be better determined by a qualitative approach, like ANOVA.

217   In this respect, log-transformation and log reduction were used as a means to calculate a standard

218   efficiency index that was independent from the initial mite count and less affected by the outliers.

219   A second output of a multifactorial ANOVA is the decomposition of the statistical hypothesis; as

220   reported elsewhere (**Bevilacqua *et al*., 2017**), the decomposition does not show actual values or effective

221   trends, but a qualitative correlation on how each predictor acts on the dependent variable (log reduction

222   of the number of *D. gallinae*). Concerning the effect of position (**Fig. 3A**), the highest mean reduction

223   was found for Block A (2.1-2.3 log-reduction). In the buffer block (Block B), the two lines experienced a

224   slight difference (1.5 log-reduction for the line 1 and 1.2 log-reduction for the line 2). Finally, in the

225   control block (Block D), the mean reduction was 0.8 log-mite (P<0.01).

226 The effect of the predictor time (**Fig. 3B)** suggests that the population of *D. gallinae* experienced a

227 decrease throughout time with the maximum reduction achieved after 59 days (P<0.01). **Fig. 3C**

228 combines the predictor position and time and shows the log-reduction for each line in each block

229 throughout time. In the treated block (A), the mean of mite-reduction was >90% after 3 days, then it

230 increased to 99% or more. After 3 days, the mean log-reduction was 40-63% in the control and buffer

231 blocks (D and B); thereafter, it increased and was >90% in the buffer block after 18 days (P<0.05).

232 An increase in log-reduction was also recovered in the control block (D), due to the main effect of the

233 predictor time and to a decrease of mite population independently from the treatment. In this block, a

234 mean effect of 90% (1-log reduction) was found after 41 days; moreover, the log-reduction for this block

235 was always lower than the values found for the buffer and the treated blocks.

236 As indices of the effect of Neem on the mites, the log-reduction after the 1st, 2nd and 3rd treatment was

237 evaluated: it was 94.65%, 99.64% and 99.80% in the treated block (Block A), 59.93%, 75.68% and

238 83.68% in the buffer (Block B) and 63.24%, 80.02% and 82.27% in the control (Block D).

239 **Fig. 4** shows more intuitively the evolution of *D. gallinae* throughout time. As reported elsewhere, the

240 mite population experienced a reduction throughout time in all the blocks; however, the rate of

241 population decrease (0.36 log mite/day in the treated Block A *vs* 0.25 log mite/day in the control and

242 buffer blocks, P at 0.023) and the residual population (0.75 log mite in the treated Block A, 2.09 log mite

243 in Block B and 3.77 log mite in Block D) support a significant effect of the neem oil in controlling *D.*

244 *gallinae* (where P=0.0001).

245

246 *Hens' response to treatment*

247 One hundred and seventy six birds, i.e., 0.9 % of the total number of hens present, died during the course

248 of the study. This figure is below the normal mortality rate for Hy-line Brown and Hy-line White hens of

249 the age used, which is 0.3-0.5% of the flock per month. Seven animals died prior to the application of

250 treatment. *Post-mortem* examination performed on all birds showed no unusual causes of death. Chronic

251 respiratory syndrome characterized by aerosacculits, catarrhal ovary and oviduct inflammation,

252 caseous peritonitis, caused by *E. coli* and/or *Mycoplasma*, were the most frequently observed causes of

253 death. Other deaths were due to accidental injuries. In no instance was any mortality event deemed

254 treatment related.

255

256 **Discussion**

257 This study is the first to investigate neem efficacy in laying hens housed within an enriched colony

258 system and supports that RP03^{TM} neem-based product is highly effective against *D. gallinae*. The product

259 caused a very high reduction of the mite population, this exceeding 99% following the second treatment,

260 and with long-lasting effects.

261 The results of mite trapping before the trial demonstrated that the *D. gallinae* population was not

262 uniformly distributed across cage blocks. Differences in number of mites registered in one block

263 compared to another were not completely unexpected, and they could be related to uncontrollable

264 variables present in the laying system, such as location, humidity, air-flow, temperature, hen breed, etc.

265 **(Nordenfors & Höglund, 2000; Arkle *et al*., 2004)**. Pre-existing differences in mite burden between

266 control and treated blocks may be considered a limitation in the present study, as differences in the initial

267 number of mites (i.e. a higher mite burden in the control block) could have potentially affected the output

268 of statistical analyses. This event could not be avoided due to a number of factors, such as the limited

269 availability of study sites and suitable facility design, intrinsic mite population variability within each

270 facility, and inevitable lag times occurring between trap collection and assessment of trap contents.

271 Because of the above, it was necessary to pre-set treatment block locations based on spatial arrangement

272 alone and not on mite counts parameters (**Fig. 1**).

273 Nevertheless, to overcome this bias and avoid the effect of a possible intrinsic variability of each block, a

274 preliminary standardization was done, by using the initial values as a baseline or internal reference for

275 each control. This approach relies on the fact that an input factor (i.e. the use of neem oil in this study)

276 affects the trend of the statistical population, but with the effect of the trend being independent from the

277 initial value (**Bevilacqua *et al*., 2016**).

11

278   Treatment with neem-based product provided a thousand-fold reduction of the mite population after the

279   second treatment (99.64%) in the current study, this reaching 99.80% after the third treatment. Even after

280   the first treatment alone, a 94% reduction in the mite population in treated blocks was observed. In

281   addition to this strong acaricidal effect and rapid knockdown of *D. gallinae*, the effect of treatment

282   persisted for more than two months.

283   The reduction rate of the mite population was significantly higher for the treated block ($P<0.001$)

284   compared to the buffer and control blocks. Nevertheless, it was also possible to observe a reduction in the

285   population of the latter two blocks over the study duration. Though this could potentially be explained by

286   the above mentioned fluctuations in environmental conditions, which are well known to affect *D.*

287   *gallinae* population density (**Nordenfors & Höglund, 2000; Arkle *et al*., 2004**), it is also possible that

288   the dispersal of RP03^{TM}, due to the forced ventilation in the unit, contributed to reduce the number of

289   mites in the blocks adjacent to the treated one, this being supported by the fact that the reduction seen

290   was stronger nearer to the treated block. Trap position was the most significant variable, as well as the

291   interactive term time/trap position. Trap position showed a mean mite log-reduction of ca. 2.2-2.4 for the

292   treated block, while in the control and buffer areas the mean reduction was 0.8 and 1.3, respectively.

293   These results were independent from the effect of time and suggest a strong bioactivity of neem.

294   After the first, the second and the third treatment, no side effects of neem were observed on laying hens,

295   with no birds displaying anomalous behavior. Furthermore, anecdotal evidence provided by the poultry

296   unit owner supported that no decrease in egg production was apparent post-treatment. Negative effects

297   were, however, reported on the equipment (conveyor belt, and cage structures), on the floor and, more

298   importantly, on eggs. The presence and the persistence of an oily film were observed for about 20 days

299   after the third treatment, while a characteristic smell tainted the eggs laid in the 24 hours after treatment,

300   likely due the contamination of the conveyor belt. Such side effects could be mitigated, at least partially,

301   by using a reduced volume of solution, or by reducing the size of the aerosol droplets**.** Reduced repeat

302   treatment schedules could also be of benefit in minimising negative effects. Due to the reclusive life cycle

303   of *D. gallinae*, repeat application of up to three times in a week is often recommended (**Abel-Gaffar *et**

304 *al.*, **2009; Locher** *et al.*, **2010**) to ensure that the generation emerging from hard-to-treat refugia post-

305 initial treatment is targeted along with any existing nymphs and adults (**George** *et al.*, **2010).** However,

306 given the high efficacy (>99%) of RP03^{TM} after the second treatment, two treatments in a week might be

307 considered as sufficient.

308 Worldwide, control of *D. gallinae* infestation is based almost exclusively on the use of synthetic

309 acaricides. Despite more than 35 molecules having been tested for use against *D. gallinae* (including

310 organophosphates, pyrethrins, pyrethroids, carbamates and amitraz), in practice, only a few products are

311 licensed in the EU for use against this pest (**Sparagano** *et al.*, **2014**). Perhaps as a consequence, several

312 unlicensed or even banned (i.e. carbaryl) products are still widely used to fight infestations in some

313 European countries (**Sparagano** *et al.*, **2014**). Recently, for example, mass recall of eggs across Europe

314 and Asia occurred due to fipronil contamination, resulting in investigations into misuse/illegal use of this

315 product by pest control to target *D. gallinae* (https://www.food.gov.uk/news-

316 updates/news/2017/16463/update-on-fipronil-in-eggs), which involved also Italy

317 (http://www.salute.gov.it/portale/news/p3_2_1_1_1.jsp?lingua=italiano&menu=notizie&p=dalministero

318 &id=3058). To promote improved product use, there is an urgent need to identify alternative, cost-

319 effective and efficacious control strategies. Among the natural compounds of use to this end (**Sparagano**

320 *et al.*, **2014; George** *et al.*, **2014),** *in vivo* experiments using neem-impregnated cardboard traps have

321 been shown to reduce *D. gallinae* populations by more than 90% (**Lundh** *et al.*, **2005**) and a neem

322 registered product (MiteStop®), diluted at 1:33 with tap water, not only killed all stages of *D. gallinae*,

323 but also did so more effectively than the synthetic organophosphate phoxim (**Abdel-Gaffar** *et al.*, **2009**).

324 Given that prolonged efficacy was registered at 162 days post-treatment in the current study (up to 90%

325 in the treated block), RP03^{TM} appears to deliver significant residual control of *D. gallinae* (i.e. of at least

326 3 months).

327

328 **Conclusion**

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

330   against *D. gallinae* in enriched colony cages. For its characteristics of safety for animals and humans

331   (**Biswas *et al.*, 2002**), azadirachtin-based products, and in particular the patented RP03^{TM}-product tested

332   here, can be suggested for *D. gallinae* control, not only in the poultry sector, but also in private and

333   public settings (residences, hospital, offices). Nevertheless, further studies should be undertaken to

334   reduce the treatment schedule, optimise the neem oil concentration and consistency and independently

335   confirm product safety. Such research should help to guarantee a high efficacy, high safety and long-

336   lasting neem acaricide, overcoming potentially undesirable effects of the registered product on poultry

337   equipment and eggs.

338

339   *Ethical statement*

340   The experiment described was authorized by the Ethical Committee for Animal Welfare of the University

341   of Foggia (Prot. n. 004-2016). The treatments did not cause detriment to the birds, and no animals were

342   sacrificed. The health and welfare conditions of the flock were assessed by independent expert veterinary

343   personnel to ensure that animals did not receive any kind of damage of suffering during and after this

344   study.

345

352

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354

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438

439   **FIGURE LEGENDS**
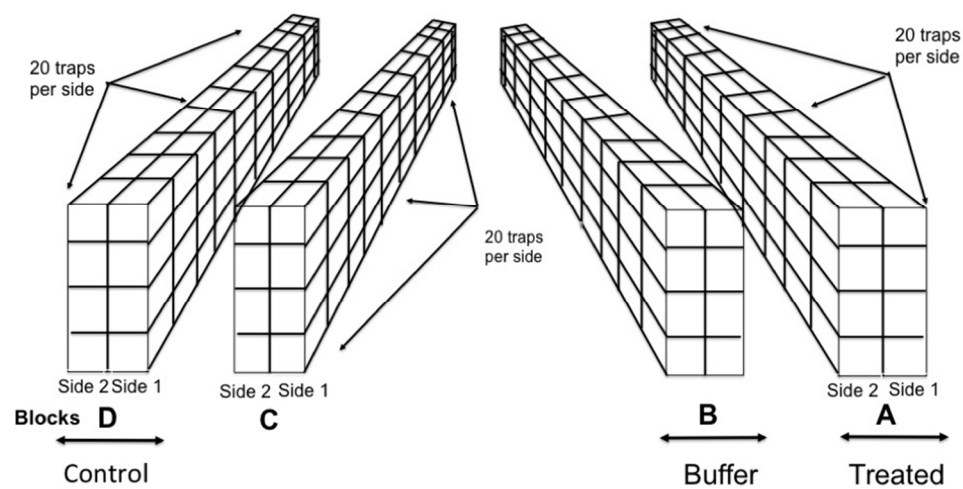
440

441   **Fig. 1**. Schematic representation of the experimental design used to test *in vivo* acaricidal activity of

442   neem-based RP03^{TM} against *Dermanyssus gallinae*. The farm building was arranged in four blocks (A-D)

443   of cages, each consisting of two adjacent lines of cages arranged over four tiers of 29 cages each

444   (providing 116 cages per block and 464 cages in total). Traps were placed in an alternating pattern on

445   each tier and each line.

446

447   **Fig. 2.** Variability plot for the population of *Dermanyssus gallinae* throughout time in the control (block

448   D) (A), buffer (block B) (B) and treated (block A) (C). The points indicate the log value for each trap, the

449   line shows the median value of each block.

450

451   **Fig. 3.** Decomposition of the statistical hypothesis for the predictors on the multifactorial ANOVA. A)

452   Effect of the position; B) Effect of time; C) Effect of the interaction position/time. The bars indicate the

453   95%-confidence intervals.

454

455   **Fig. 4.** Evolution of *Dermanyssus gallinae*. k_{max} = rate of population decrease; $N_{res,}/$ = survivors (mean

456   values ± standard error). T1 = 1st treatment; T2, 2nd treatment; T3, 3rd treatment.

457   The population evolution is fitted up to 87 days, though the last point shown indicates the mean values of

458   the mite population after 162 days.

459

460   **Supporting Information files**

461   **Table S1.** Scheme of the trial schedule

462   **Table S2.** Number of *Dermanyssus gallinae* registered throughout the trial in Treated (A), Buffer (B) and

463   Control (D) blocks, on one side of the block line (1), on the other side of the block line (2) and average

464   on both lines (mean value of 1 and 2).

19

465
466

467

20

1   **Table 1.** Standardized effects of the multifactorial ANOVA. The analysis was run by using the GLM

2   option in Statistica; the standard error of the model was 0.53 log *Dermanyssus gallinae*.

3

	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		

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

5

6

1

Fig. 1. Schematic representation of the experimental design used to test in vivo acaricidal activity of neem-based RP03TM 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.
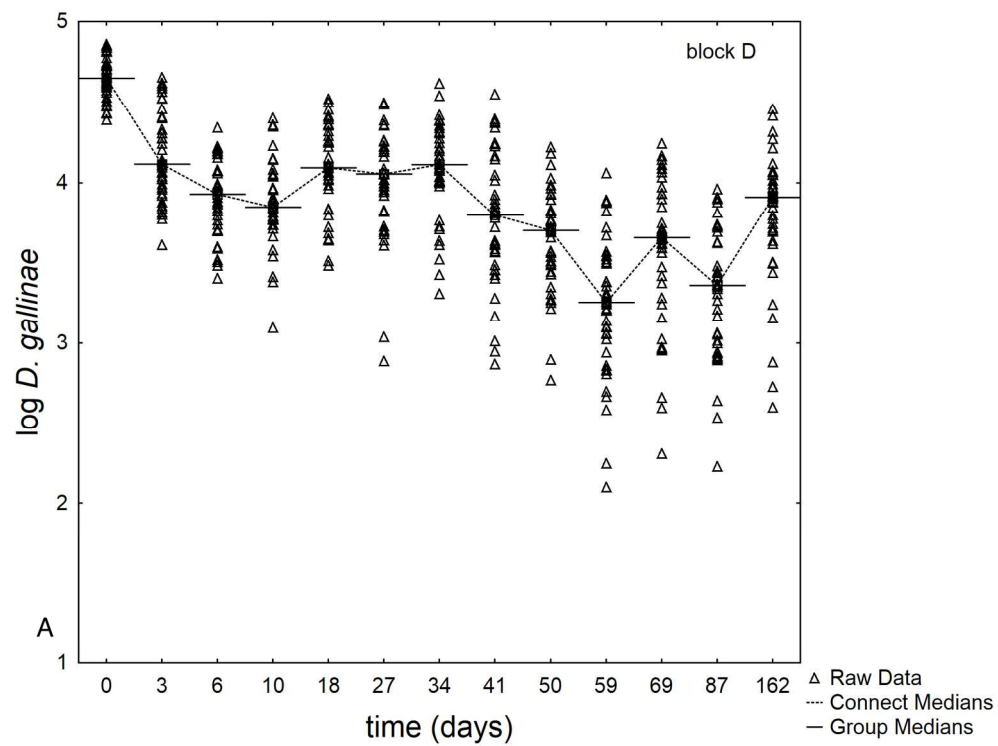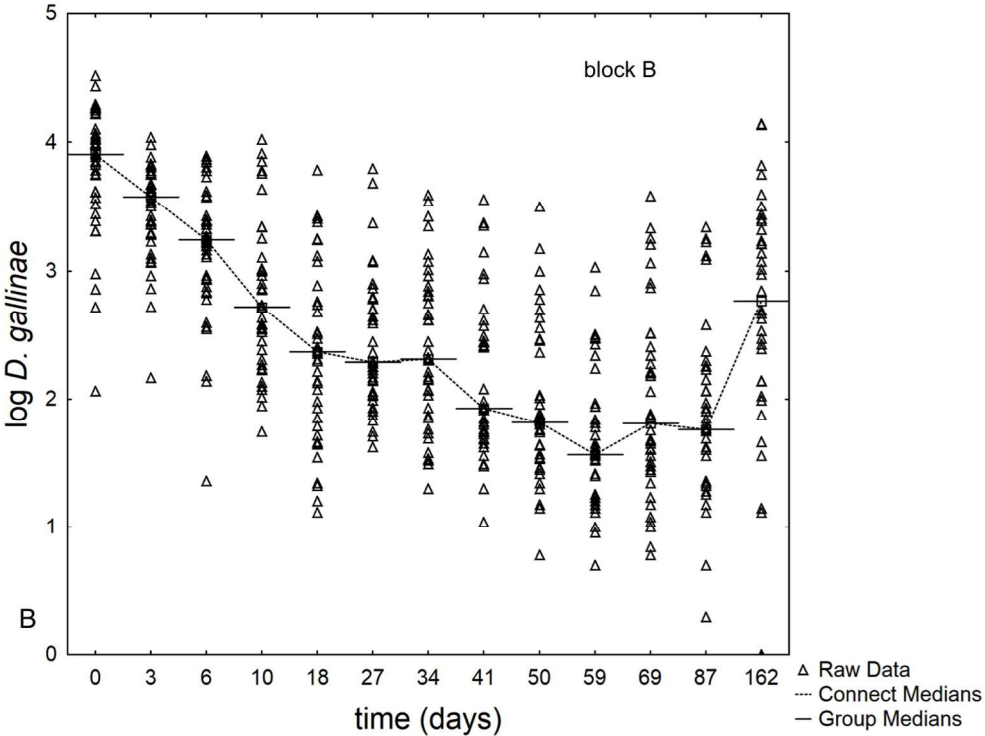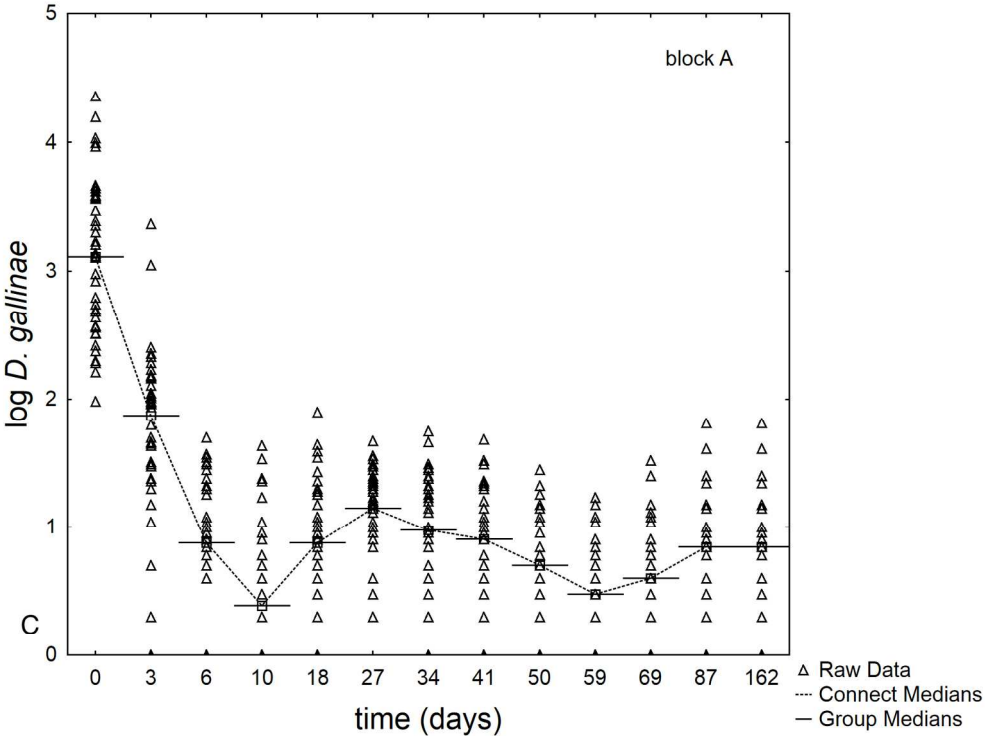
170x81mm (150 x 150 DPI)

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)
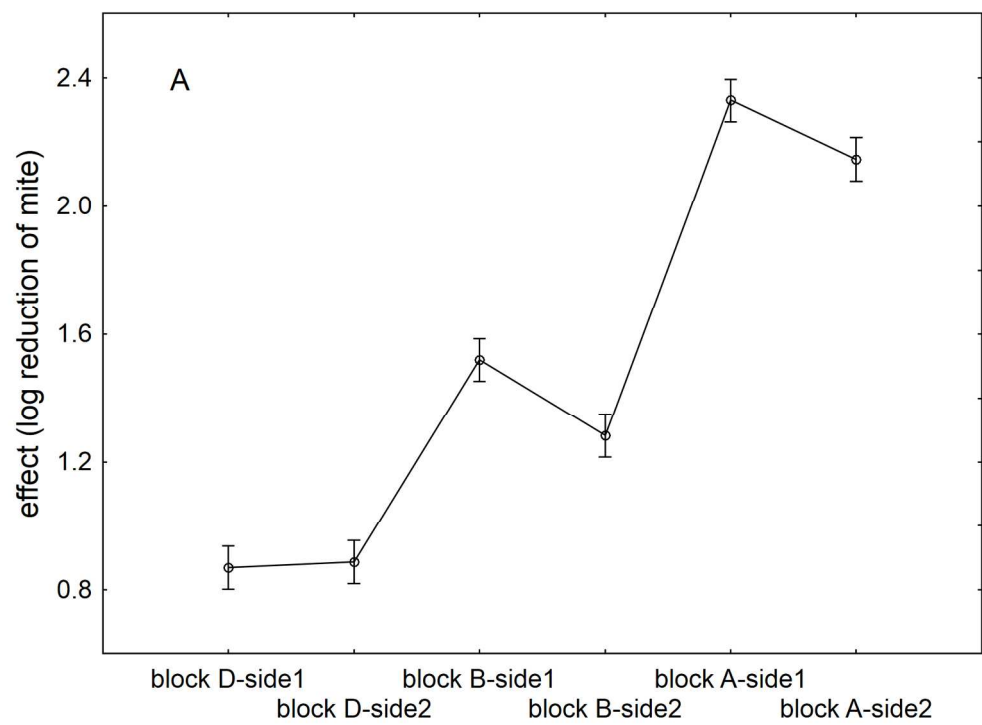
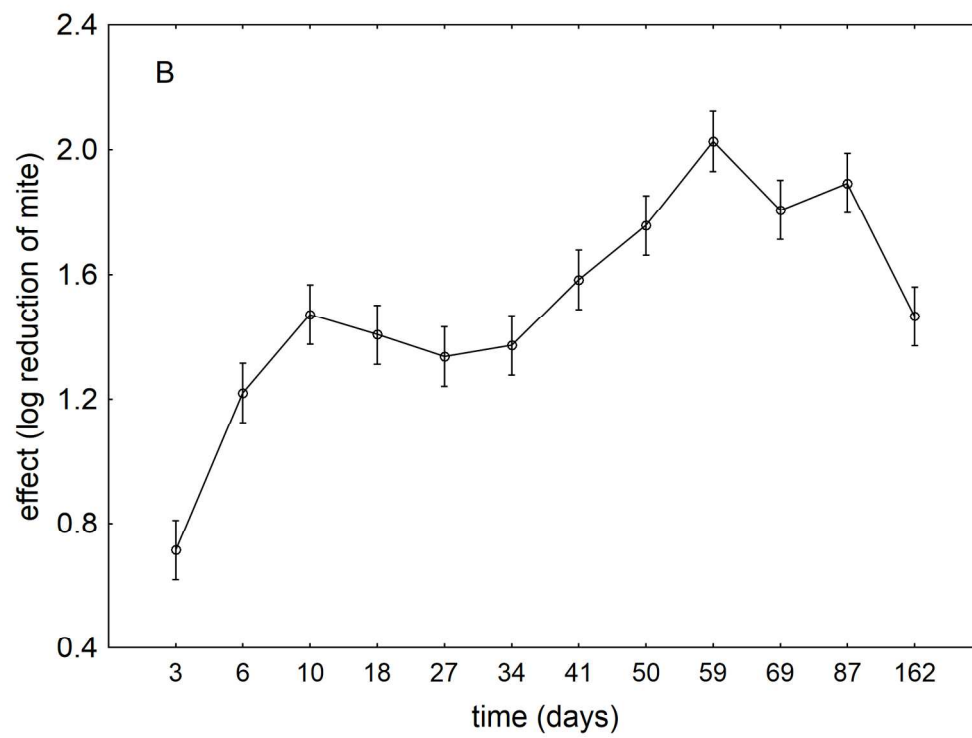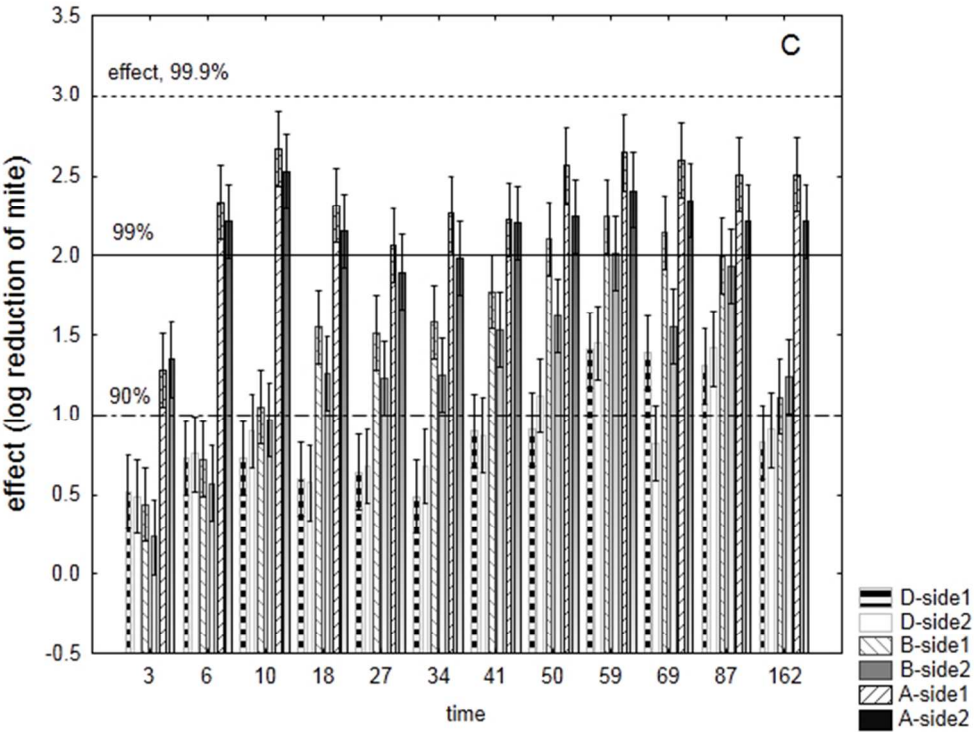254x190mm (150 x 150 DPI)

515x387mm (96 x 96 DPI)

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)

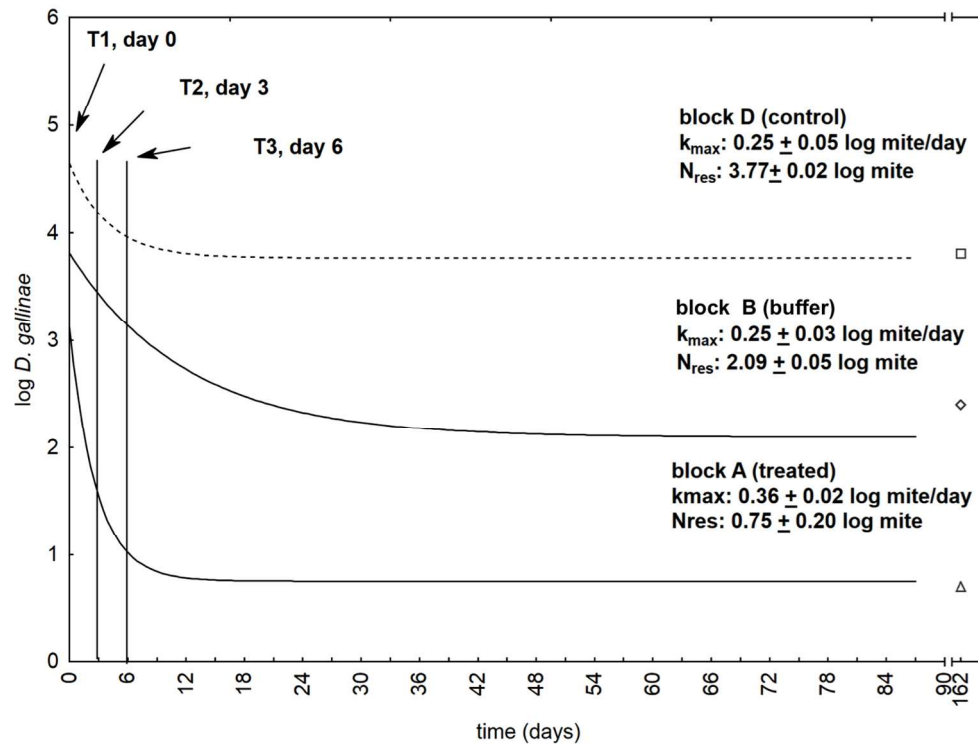515x387mm (96 x 96 DPI)

165x123mm (96 x 96 DPI)

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)

1   **Table S1.** Scheme of the trial schedule

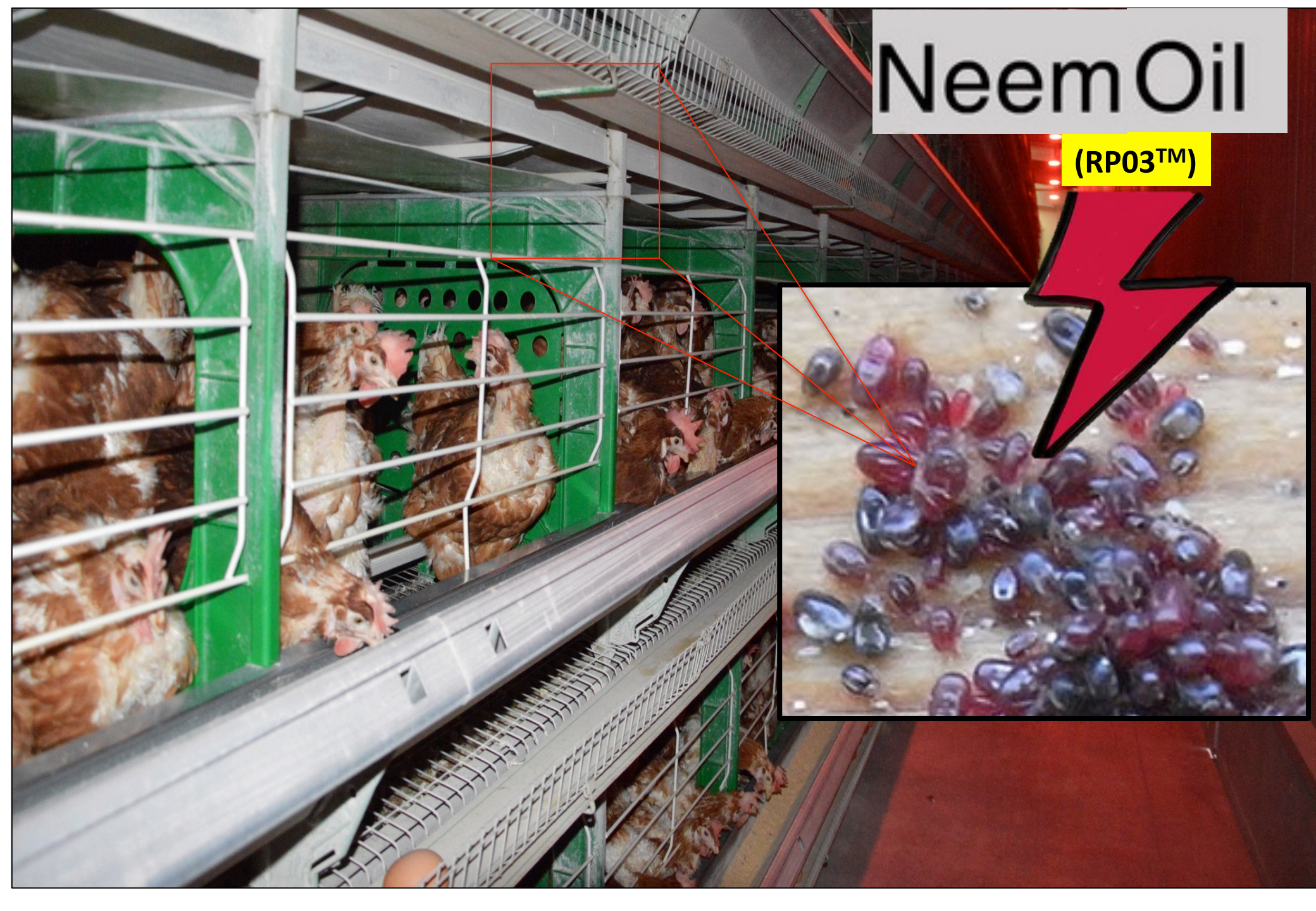| Day	Events
-3	Placement of traps
0 (T^1)	Cardboard removal and count **First treatment with RP03^{TM}** and Cardboard replacement
3 (T^2)	Cardboard removal and count, **Second treatment with RP03^{TM}** and Cardboard replacement
6 ($T^{3)}$	Cardboard removal and count **Third treatment with RP03^{TM}**
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

2
3   T^1: 1st treatment; T^2: 2nd treatment; $T^{3:}$ 3rd treatment
4

1

1   **Table S2.** Number of *Dermanyssus gallinae* registered throughout the trial in Treated (A), Buffer (B) and

2   Control (D) blocks, on one side of the block line (1), on the other side of the block line (2) and average

3   on both lines (mean value of 1 and 2)

4

Days	Mite mean count ± SD								
	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

5

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

Twitter

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