Combined effect of the entomopathogenic fungus *Metarhizium robertsii* and avermectins on the survival and immune response of *Aedes aegypti* larvae

Yuriy A Noskov [Corresp., 1, 2] , Olga V Polenogova [1] , Olga N Yaroslavtseva [1] , Olga E Belevich [1] , Yuriy Y Yurchenko [1] , Ekaterina A Chertkova [1] , Natalya A Kryukova [1] , Vadim Y Kryukov [1] , Viktor V Glupov [1]

[1] Institute of Systematics and Ecology of Animals, Novosibirsk, Russian Federation

[2] Tomsk State University, Tomsk, Russian Federation

Corresponding Author: Yuriy A Noskov
Email address: yunoskov@gmail.com

Combination of insect pathogenic fungi and microbial metabolites is a prospective method for mosquito control. The effect of the entomopathogenic fungus *Metarhizium robertsii* and avermectins on the survival and physiological parameters of *Aedes aegypti* larvae (dopamine concentration (DA), glutathione S-transferase (GST), nonspecific esterases (EST), acid proteases, lysozyme-like, phenoloxidase (PO) activities) was studied. It is shown that the combination of these agents leads to a synergistic effect on mosquito mortality. Systemic colonization of the larvae of *Ae. aegypti* by hyphal bodies following water inoculation with conidia is shown for the first time. The larvae affected by fungi are characterized by a decrease in PO and dopamine levels. In the initial stages of toxicosis and/or fungal infection (12 h posttreatment), increases in the activity of insect detoxifying enzymes (GST and EST) and acid proteases are observed after monotreatments, and these increases are suppressed after combined treatment with the fungus and avermectins. Lysozyme-like activity is also most strongly suppressed under combined treatment with the fungus and avermectins in the early stages posttreatment (12 h). Forty-eight hours posttreatment, we observe increases in GST, EST, acid proteases, and lysozyme-like activityunder the influence of the fungus and/or avermectins. The larvae affected by avermectins accumulate lower levels of conidia than avermectin-free larvae. On the other hand, a burst of bacterial CFUs is observed under treatment with both the fungus and avermectins. We assume that disturbance of the responses of the immune and detoxifying systems under the combined treatment and the development of opportunistic bacteria may be among the causes of the synergistic effect.

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2 **the survival and immune response of *Aedes aegypti* larvae**

4 Yuriy A. Noskov[1,2], Olga V. Polenogova[1], Olga N. Yaroslavtseva[1], Olga E. Belevich[1], Yuriy A.
5 Yurchenko[1], Ekaterina A. Chertkova[1], Natalya A. Kryukova[1],Vadim Y. Kryukov[1], Viktor V.
6 Glupov[1]

8 [1] Institute of Systematics and Ecology of Animals SB RAS, Novosibirsk, Russia

9 [2] National Research Tomsk State University, Tomsk, Russia

11 Corresponding author:

12 Yuriy Noskov [1,2]

13 Frunze str., 11, Novosibirsk, 630091, Russia

14 Email address: yunoskov@gmail.com

16   **Abstract**
17   Combination of insect pathogenic fungi and microbial metabolites is a prospective method for
18   mosquito control. The effect of the entomopathogenic fungus *Metarhizium robertsii* and
19   avermectins on the survival and physiological parameters of *Aedes aegypti* larvae (dopamine
20   concentration (DA), glutathione S-transferase (GST), nonspecific esterases (EST), acid
21   proteases, lysozyme-like, phenoloxidase (PO) activities) was studied. It is shown that the
22   combination of these agents leads to a synergistic effect on mosquito mortality. Systemic
23   colonization of the larvae of *Ae. aegypti* by hyphal bodies following water inoculation with
24   conidia is shown for the first time. The larvae affected by fungi are characterized by a decrease
25   in PO and dopamine levels. In the initial stages of toxicosis and/or fungal infection (12 h
26   posttreatment), increases in the activity of insect detoxifying enzymes (GST and EST) and acid
27   proteases are observed after monotreatments, and these increases are suppressed after combined
28   treatment with the fungus and avermectins. Lysozyme-like activity is also most strongly
29   suppressed under combined treatment with the fungus and avermectins in the early stages
30   posttreatment (12 h). Forty-eight hours posttreatment, we observe increases in GST, EST, acid
31   proteases, and lysozyme-like activityunder the influence of the fungus and/or avermectins. The
32   larvae affected by avermectins accumulate lower levels of conidia than avermectin-free larvae.
33   On the other hand, a burst of bacterial CFUs is observed under treatment with both the fungus
34   and avermectins. We assume that disturbance of the responses of the immune and detoxifying
35   systems under the combined treatment and the development of opportunistic bacteria may be
36   among the causes of the synergistic effect.

37

38   **Introduction**
39   Mosquitoes are obligate intermediate hosts for a variety of pathogens that cause human mortality
40   and morbidity worldwide. *Aedes aegypti* is considered to be an important vector of human
41   diseases such as dengue and yellow fever, chikungunya, and Zika infections (*Tolle, 2009; Bhatt*
42   *et al., 2013*), and its control is therefore an objective to prevent the transmission of these
43   diseases. Chemical insecticides are still the most important element in mosquito control
44   programs, despite direct and indirect toxic effects on nontarget organisms, including humans. In
45   addition, chemicals induce resistance in a number of vector species (*Hemingway & Ranson,*
46   *2000; Vontas, Ranson & Alphey, 2010*). Therefore, there is a need for alternative nonchemical
47   vector control approaches. Classical biological control based on using various microorganisms
48   such as entomopathogenic fungi and bacteria is a frequent tool for addressing this issue.
49   Among the biological agents employed for mosquito larvae control, bacteria from the genus
50   *Bacillus* are the most widely used. In addition, products of the entomophatogenic fungi (EPF)
51   Metarhizium and Beauveria are actively being developed for use against mosquito adults and
52   larvae (*Butt et al., 2013; Greenfield et al., 2015; Ortiz-Urquiza et al., 2015*). It should be noted
53   that mosquitoes and other insects can develop resistance to the *Bacillus thuringiensis* biological
54   larvicide (*Tilquin et al., 2008; Paris et al., 2011; Boyer et al., 2012*). However, the resistance of
55   insects to entomopathogenic fungi develops very slowly (*Dubovskiy et al., 2013*). Various
56   species of mosquito larvae present different susceptibilities to Metarhizium, among which *Ae.*
57   *aegypti* is the least susceptible (*Greenfield et al., 2015; Garrido-Jurado et al., 2016*). Thus, a
58   concentration of conidia that is effective for *Ae. aegypti* control would affect a range of nontarget
59   aquatic invertebrates. Recent studies have found that some nontarget aquatic species are more
60   sensitive to fungal metabolites (*Garrido-Jurado et al., 2016*) and conidia (*Belevich et al., 2017*)
61   than target mosquito species. To reduce toxic effects on the aquatic environment and increase

62 efficacy against mosquitoes, entomopathogenic fungi may be combined with other biocontrol
63 agents or low doses of natural insecticides. For example, combined treatment with Metarhizium
64 and mosquito predator species (*Toxorhynchites*) has shown additive or synergistic effects on the
65 mortality of *Ae. aegypti* (*Alkhaibari et al.,* 2018). However, few studies have been carried out to
66 determine the effect of combined treatment with EPF and other insecticides or plant or microbial
67 metabolites as a potential tool for improving mosquito larvae control. Synergistic effects
68 between EPF and some chemical insecticides (temephos, spinosad) (*Shoukat et al., 2018;*
69 *Vivekanandhan et al., 2018*) or biological agents (*Azadirachta indica*) (*Badiane et al., 2017*) on
70 the mortality of mosquito larvae have been found. However, the physiological and biochemical
71 aspects of this synergism were not considered.
72 One type of promising insecticide that can be effectively used for mosquito vector control is he
73 avermectins. Avermectins are a class of macrocyclic lactones isolated from the soil actinomycete
74 *Streptomices avermitilis* (*Drinyaev et al., 1999*) and include several commercial derivatives
75 (ivermectin, abamectin, doramectin and eprinomectin) with the same mode of action – activation
76 of glutamate-gated chloride channels, followed by uncontrolled influx of chloride ions into the
77 cells, which leads to paralysis and death of the organism (*Campbell et al., 1983)*. At the same
78 time, avermectins are relatively safe for humans (*Crump, Omura, 2011*). Previous studies have
79 shown that avermectins are efficient for the control of *Culex quinquefasciatus* (*Freitas et al.,*
80 *1996; Alves et al., 2004*), *Anopheles albimanus* and *An. stephensi* (*Dreyer, Morin & Vaughan,*
81 *2018*), and *An. gambiae* (*Alout et al., 2014; Chaccour et al., 2017*). However, most of these
82 studies have been carried out with adult mosquitoes feeding on blood containing ivermectin.
83 Avermectins exhibit a relatively short half-life period, which limits their ability to kill
84 mosquitoes but may be compensated by the application of multiple treatments or use of higher
85 concentrations. However, these approaches may contribute to the development of mosquito
86 larvae resistance to avermectins (*Su et al., 2017)*. We hypothesize that the interaction of EPF
87 with avermectins can have a stable insecticidal effect at relatively low concentrations and is a
88 promising combination for safe and effective mosquito control.
89 It is important that EPF such as Metarhizium are adapted to terrestrial hosts and that in mosquito
90 larvae, the fungi do not adhere to the cuticle surface and do not germinate through integuments
91 into the body cavity. Conidia ingested by mosquito larvae do not penetrate the gut wall (*Butt et*
92 *al., 2013*). Thus, a "classic" host-pathogen interaction does not occur, and larval mortality is
93 associated with stress induced by spore-bound proteases on the surface of ingested conidia (*Butt*
94 *et al., 2013*). These authors suggest that fungal proteases cause an increase in the activity of
95 caspases in mosquitoes, which leads to apoptosis, autolysis of tissues and death of the larvae.
96 The activation of detoxifying enzymes and antimicrobial peptides (AMPs) occurs in larvae
97 infected with the fungus but is not sufficient to protect the larvae from death. As a rule, mosquito
98 larvae die showing symptoms of bacterial decomposition after treatment with Metarhizium and
99 Beauveria (*Scholte et al., 2004*). Therefore, this pathogenesis can be considered mixed (both
100 bacterial and fungal).
101 During this process, particular superfamilies of enzymes such as glutathione-S-transferases
102 (GST) and nonspecific esterases (EST) are usually involved in the biochemical transformation of
103 xenobiotics. Various hormones such as biogenic amines are involved in insect stress reactions.
104 Among these hormones, the role of the neurohormone dopamine (DA) in this process remains
105 poorly understood. It is known that DA mediates phagocytosis and is involved in the activation
106 of the pro-phenoloxidase (proPO) cascade, thus playing an important role in fungal and bacterial
107 pathogenesis as well as in the development of toxicoses caused by insecticides (*Delpuech, Frey*

108   *& Carton, 1996; Gorman, An & Kanost, 2007; Wu et al., 2015*). In addition, both pathogens and
109   toxicants can lead to changes in the antimicrobial activity of insects and the bacterial load that
110   can affect the sensitivity of insects to pathogens (*Wei et al., 2017; Ramirez et al., 2018;*
111   *Polenogova et al., 2019*). It should be noted that the above mentioned physiological reactions in
112   mosquito larvae under the combined action of entomopathogenic fungi and insecticides have not
113   yet been studied.
114   The aims of this study were (1) to determine the susceptibility of *Aedes aegypti* larvae to
115   combined treatment with avermectins and *Metarhizium robertsii* J.F. Bischoff, S.A. Rehner &
116   Humber and (2) estimated their immune and detoxificative responses to *M. robertsii* and
117   avermectins either alone or their combination.
118
119   **Materials & methods**
120
121   **Insecticides and fungi**
122   The entomopathogenic fungus *Metarhizium robertsii* (strain P-72; GenBank No. KP172147)
123   from the collection of microorganisms of the Institute of Systematics and Ecology of Animals
124   SB RAS was used in this work. The conidia of the fungus were grown on autoclaved millet for
125   10 days at 26°C in the dark, followed by drying and sifting (*Belevich et al., 2017*). The industrial
126   product "Phytoverm" 0.2 % (SPC "Pharmbiomed", Russia) was used in these experiments and
127   includes a complex of natural avermectins (A1a (9 %), A2a (18 %), B1a (46 %), B2a (27 %))
128   produced by *Streptomyces avermitilis*.
129
130   **Insect maintenance and toxicity tests**
131   *Aedes aegypti* larvae from the collection of the Institute of Systematics and Ecology of Animals
132   SB RAS were maintained in tap water in the laboratory at 24°C (±1°C) under a natural
133   photoperiod (approximately 16:8 light:dark). The larvae were fed Tetramin Junior fish food
134   (Tetra, Germany). The susceptibility of *Ae. aegypti* to both avermectins and the conidia of *M.*
135   *robertsii* was tested in 200 ml plastic containers containing 100 ml of water with 15 larvae.3rd-
136   4th-instar larvae were used in the experiment.The experiment involved four treatments: control,
137   fungus, avermectins, and fungus + avermectins. The fungal conidia and avermectins were
138   suspended (or dissolved) in distilled water, vortexed and applied separately or together to the
139   containers with mosquito larvae at a volume of 2 ml per container. The final conidial
140   concentration for infection was 1×10^6 conidia / ml ($\approx$ LC50). The final concentration of
141   avermectins was 0.00001 % / ml ($\approx$ LC50). The control was treated with the same amount of
142   distilled water. Mortality was assessed daily for 6 days. Ten replicates with 15 larvae were
143   performed for each treatment.
144
145   **Light microscopy and colonization assessment**
146   Forty-eight hours posttreatment (pt), mosquito larvae (n = 3 for each treatment) were collected in
147   2% glutaraldehyde containing 0.1 M Na-cacodylate buffer (pH 7.2) and were maintained at 4 °C
148   for 1–24 h. Semithin sections were stained with crystal violet and basic fuchsin and were
149   observed with a phase-contrast microscope (Axioskop 40, Carl Zeiss, Germany).
150   To assess fungal colonization, 48 h pt and newly dead larvae (4–6 days pt) were cut open, and
151   their internal contents were squeezed onto a glass side. The contents were examined for the
152   presence / absence of hyphal bodies using light microscopy (n = 30 for each treatment). Newly
153   dead larvae were placed on moistened filter paper in Petri dishes (n = 30) to determine the

154 germination and surface sporulation of Metarhizium.
155
156 **Total larval body supernatant**
157 Mosquito larvae bodies of individual 4th-instar larvae of *Ae. aegypti* were collected in 50 μl of
158 cool (+4°C) 0.01M PBS (50 mM, pH 7.4, 150 mM NaCl) with 0.1 mM N-phenylthiourea (PTU)
159 to measure GST or without PTU to measure phenoloxidase (PO) activity. Then, the samples
160 were sonicated in an ice bath with three 10 s bursts using a Bandelin Sonopuls sonicator. The
161 sample solution was centrifuged at 20.000 g for 5 min at +4°C. The obtained supernatant was
162 directly used to determine enzyme activities.
163
164 **Detection of phenoloxidase, glutathione-S-transferase and esterase activity**
165 The activities of PO, GST and EST were measured at 12 and 48 h after exposure (n = 20 per
166 treatment for each enzyme).
167 PO activity was assayed by using a method modified from that described by Ashida and
168 Söderhäll (*1984*). The PO activity of the larval homogenates was determined
169 spectrophotometrically on the basis of the formation of dopachrome at a wavelength of 490 nm.
170 Aliquots of the samples (10 μl) were added to microplate wells containing 200 μl of 10 mM 3.4-
171 dihydroxyphenylalanine (DOPA) and incubated at 28 ºC for 45 min.
172 The activity of EST was measured using the method of Prabhakaran and Kamble (*1995*) with
173 some modifications. Aliquots of the samples (3 μl) were added to microplate wells containing
174 200 μl of 0.01 % p-nitrophenylacetate and incubated for 5 min at 28 °C. The activity of EST was
175 determined spectrophotometrically at a wavelength of 410 nm on the basis of the formation of
176 nitrophenyl.
177 The measurement of GST activity was carried out according to the method of V. Habig et al.
178 (*1974*) with some modifications. Aliquots of the samples (5 μl) were added to microplate wells
179 containing 200 μl of 1 mM glutathione and 5 μl of 1 mM DNCB and incubated at 28 °C for 20
180 min. The activity of GST was determined spectrophotometrically on the basis of the formation of
181 5-(2.4-dinitrophenyl)-glutathione at a 340 nm wavelength.
182 Enzyme activity was measured in units of the transmission density (ΔA) of the incubation
183 mixture during the reaction per 1 min and 1mg of protein. The protein concentration in the
184 samples was determined by the method of M. Bradford (*1976*). To generate the calibration curve,
185 bovine serum albumin was used.
186
187 **Dopamine concentration measurements**
188 DA concentrations were measured at the 12 and 48 h pt in individual larval bodies (n = 10 per
189 treatment). Mosquito larvae were homogenized in 30 μl of phosphate buffer and incubated in a
190 Biosan TS 100 Thermoshaker for 10 minutes at 28 °C and 600 rpm, then incubated at room
191 temperature for 20 minutes and centrifuged at 4 °C and 10.000 g for 10 minutes. The
192 supernatants were transferred to clean tubes and centrifuged with the same settings for 5 minutes.
193 Before transfer to the chromatograph, the samples were filtered.
194 DA concentrations were measured by an external standard method using an Agilent 1260 Infinity
195 high-performance liquid chromatograph with an EsaCoulochem III electrochemical detector (cell
196 model 5010A, potential 300 mV) according to the method of Gruntenko et al. (2005) with some
197 modifications. Dopamine hydrochloride (Sigma-Aldrich) was used as a standard. Separation was
198 performed in a ZorbaxSB-C18 column (4.6–250 mm, particles 5 μm) in isocratic mode. Mobile
199 phase: 90% buffer (200 mg/l 1-OctaneSulfonicAcid (Sigma-Aldrich), 3.5 g/l KH_2PO_4) and 10%

200   acetonitrile. The flow rate was 1 ml/min. Chromatogram processing was performed using
201   ChemStation software, and the amount of dopamine was determined by comparing the peak
202   areas of the standard and the sample.
203
204   **Acid proteases**
205   For the measurement of acid proteases, 50 µl of the homogenate supernatant was added to 250 µl
206   of 0.1 M acetate buffer (pH 4.6) containing 0.3% hemoglobin (Sigma). The samples were
207   incubated for 60 min at 27 °C, and the reaction was stopped by adding 500 µl of 5% TCA and
208   cooling on ice for 10 min at 4 °C. The samples were centrifuged at 14000 g for 5 min at 4 °C,
209   andenzyme activity was determined spectrophotometrically at a wavelength of 280 nm in a 96-
210   well plate reader.
211
212   **Lysozyme-like activity**
213   Lysozyme-like activity in the mosquito homogenate was determined through analysis of the lytic
214   zone by diffusion into agar. Ten milliliters of Nutrient Agar (NA) (HiMedia, India) and
215   *Micrococcus lysodeikticus* bacteria (1×10^7 cells/ml) were added to Petri dishes. The agar was
216   perforated to create 2 mm-diameter wells, which were then filled with 3 µl of fresh hemolymph,
217   followed by incubation at 37 °C for 24 h. A series of dilutions of chicken egg white lysozyme
218   (EWL) (Sigma) (0.5 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.005 mg/ml, 0.001 mg/ml) were added to
219   each dish, allowing us to obtain a calibration curve based on these standards. Lytic activity was
220   determined by measuring the diameter of the clear zone around each well and expressed as the
221   equivalent of EWL (mg/ml) (*Mohrig, Messner, 1968*).
222
223   **CFU counts of Metarhizium and cultivated bacteriain infected larvae**
224   Homogenates of the mosquitoes (3 larvae per sample) were suspended in sterile aqueous Tween-
225   20 (0.03%) (1 ml), and the suspensions were then diluted 50-fold. Next, 100 µl aliquots were
226   inoculated onto the surface of modified Sabouraud agar (10 g peptone, 40 g D-glucose
227   anhydrous, 20 g agar, 1 g yeast extract) supplemented with an antibiotic cocktail (acetyltrimethyl
228   ammonium bromide 0.35 g/L; cycloheximide 0.05 g/L; tetracycline 0.05 g/L; streptomycin 0.6
229   g/L; PanReacAppliChem, Germany) for the inhibition of bacteria and saprotrophic fungi. The
230   Petri dishes were maintained at 28 °C in the dark. The colonies were then counted after 7 days.
231   For the estimation of cultivated bacterial CFU counts, homogenates of larvae (3 larvae per
232   sample) were suspended in 1ml of 0.1M phosphate buffer. Then, the suspension was diluted to
233   10^{-2}, 10^{-3} and 10^{-4}.Aliquots of 100 µl of the larval dilutions were inoculated onto the surface of
234   blood agar media (HiMedia, India). The Petri dishes were maintained at 28 ºC. The colonies
235   were counted after 48 h. Three samples of each treatment were used in the analysis.
236
237   **Statistical analysis**
238   Data were analyzed using GraphPad Prism v.4.0 (GraphPad Software Inc., USA), Statistica 8
239   (StatSoft Inc., USA), PAST 3 (*Hammer, Harper, Ryan, 2001*) and AtteStat 12.5 (*Gaidyshev,*
240   *2004*). Differences between synergistic and additive effects were determined by comparing the
241   expected and observed insect mortality using the χ^2 criterion (*Tounou et al., 2008*). An additive
242   effect was recorded at $\chi^2 < 3.84$ or a synergistic effect at $\chi^2 > 3.84$ if the observed mortality was
243   higher than expected, and an antagonistic was recorded at $\chi^2 > 3.84$ if the observed mortality was
244   lower than expected. The Kaplan-Meier test was used to calculate the median lethal time
245   (presented as LT50 ± SE). A log-rank test was used to quantify differences in mortality

246   dynamics. As the distribution of the physiological parameters except for the dopamine
247   concentration deviated from a normal distribution (Shapiro–Wilk test, P < 0.05), we used the
248   nonparametric equivalent of a two-way ANOVA: the Scheirer-Ray-Hare test (*Scheirer et al.,*
249   *1976*), followed by Dunn's post hoc test. The data on dopamine concentrations passed the
250   normality test (Shapiro–Wilk test, P > 0.05) and were analyzed by two-way ANOVA followed
251   by Tukey's post hoc test. Differences between Metarhizium CFU counts were compared by t-
252   tests.

253   **Results**
254
255   **Synergy between avermectins and the fungus**
256   Significant differences in the dynamics of larval mortality between the treatments were observed
257   (log-rank test: χ^2 = 397.3 Df = 3, P < 0.0001; Fig. 1). Treatment with avermectins or conidia of
258   *M. robertsii* led to 57 and 55% mortality, respectively, whereas combined treatment led to 99%
259   mortality at the 6th day postinfection. Mortality in the control treatment did not exceed 1%. The
260   median lethal time (LT50) post-combined treatment (3 ± 0.1 d) occurred twice as fast as under
261   treatment with avermectins (6 ± 0.3 d) or the fungus (6 d ± inf.) alone (χ^2 > 113.7 Df = 1, P <
262   0.0001).
263   From the 2nd to the 6th day pt, the avermectins and fungus interacted synergistically (χ^2 > 18.5,
264   df = 1, P < 0.001, ESM Table S1). These effects were consistently observed in four independent
265   experiments.
266
267   **Colonization assay**
268   At 48 h pt, we observed mass accumulation of Metarhizium conidia in the gut lumen (Fig. 2A).
269   Germinated conidia were not detected in larvae at 48 h pt (n = 12). However, in one sample
270   (combined treatment),hyphal bodies were detected in the hemocoel (Fig. 2A). In the newly dead
271   larvae after the fungal and combined treatments (4–6 days), we detected systemic colonization
272   with hyphal bodies (Fig. 2B). Under the combined treatment, 83% hypha-positive larvae were
273   found, while in the fungal treatment, 90% hypha-positive larvae were recorded. No significant
274   differences between these treatments were observed (χ^2 = 0.58, df = 1, P = 0.45, n = 30 larvae
275   per treatment). No hyphal bodies were detected in the fungus-free treatments. A total of 70% and
276   60 % of larvae were overgrown with Metarhizium under incubation in moist chambers (Fig. 2C)
277   after treatment with the fungus or the mixture (avermectins+fungus), respectively.
278   Nongerminated conidia and hyphal bodies were detected in the water in which treated larvae
279   were maintained (Fig. 2D).
280
281   **Phenoloxidase activity**
282   At 12 h pt, we registered a significant decrease in PO activity under the influence of fungal
283   infection (Scheirer-Ray-Haretest, effect of fungus: $H_{1.52}$ = 12.6, P = 0.00038; Fig. 3).
284   Avermectins did not significantly change PO activity ($H_{1.52}$ = 0.3, P = 0.54). A stronger decrease
285   in enzyme activity was observed after combined treatment, but a significant factor interaction
286   was not revealed ($H_{1.52}$ = 1.9, P = 0.16). At 48 h pt, we detected a significant increase in PO
287   activity under the influence of avermectins ($H_{1.32}$ = 5.33, P = 0.02). The effect of the fungus was
288   not significant ($H_{1.32}$ = 0.7, P = 0.39), but a tendency toward an interaction between the factors
289   was revealed ($H_{1.32}$ = 3.2, P = 0.07). This is explained by the inhibition of PO activity by the
290   fungus alone (Dunn's test, P = 0.01, P = 0.04, compared to the control and avermectin

291 treatments, respectively) and by the tendency of increased enzyme activity after combined
292 treatment.
293
Dopamine concentration
295 The effects of the fungus or avermectins on the DA concentration at 12 h pt were not significant
296 ($H_{1.31}$ = 1.2, P = 0.27; Fig. 4), although a trend toward a factor interaction was revealed ($H_{1.31}$ =
297 3.3, P = 0.07). This was due to a clear tendency to decrease the DA concentration after treatment
298 with the fungus alone (Dunn's test, P = 0.07 compared to fungus-free treatments) but not with
299 the combination of the fungus and avermectins (P = 0.47 compared to fungus-free treatments).
300 At 48 h pt, we observed a significant decrease in the DA concentration under the influence of the
301 fungus ($H_{1.31}$ = 4.62, P = 0.03); however, there were no significant differences between the
302 treatments (Dunn's test, p = 0.1). No significant interaction effects between the factors on the
303 DA concentration at 48 h pt were detected ($H_{1.31}$ = 0.0, P = 1.0).
304
Detoxifying enzymes
306 At 12 h pt, an interaction effect between the two factors (avermectins and the fungus) on GST
307 activity was observed ($H_{1.44}$ = 8.1, P = 0.0043, Fig. 5A). Avermectins and the fungus alone
308 significantly (1.5–2-fold) increased GST activity compared to untreated larvae (Dunn's test, P =
309 0.007, P = 0.001, respectively), but after combined treatment, the enzyme activity did not
310 significantly differ from that in the control. Similar patterns were registered for EST activity at
311 12 h pt (Fig. 5B). A trend toward an interaction between fungal infection and avermectins was
312 revealed ($H_{1.44}$ = 2.14, P = 0.1). In this case, EST was activated under the influence of
313 avermectins alone (Dunn's test P = 0.002, compared to control), but fungal infection inhibited
314 this activation. In particular, EST activity in the fungal and combined treatments did not differ
315 from that in the control (Dunn's test P = 0.28, P = 0.34, respectively).
316 At 48 h pt, we observed a significant increase in GST activity under the influence of avermectins
317 ($H_{1.32}$ = 4.2, P = 0.03). The effect of the fungus as well as the interaction between the factors on
318 GST activity at this time point was not significant ($H_{1.32}$ = 2.5, P = 0.1 and $H_{1.32}$ = 0.61, P = 0.43,
319 respectively). EST activity nonsignificantly increased under the influence of avermectins ($H_{1.32}$ =
320 1.85, P = 0.17). The effect of the fungus on enzyme activity was not significant ($H_{1.32}$ = 0.001, P
321 = 0.96), and no significant interactions between the factors were detected ($H_{1.32}$ = 0.49, P =
322 0.48).
323
Acid protease activity
325 At 12 h pt, a significant interaction between avermectins and the fungus on acid protease activity
326 was observed ($H_{1.48}$ = 14.8; P = 0.00011; Fig. 6). In particular, protease activity was increased
327 after fungal treatment alone (3-fold compared to control, P = 0.0003) but not after combined
328 treatment. At 48 h pt, protease activity was strongly increased under the influence of avermectins
329 ($H_{1.43}$ = 27.4, P = 0.000016), and a trend toward an increase in enzyme activity was registered
330 under the influence of the fungus ($H_{1.43}$ = 3.18, P = 0.07). No significant interaction between the
331 factors was revealed, although a trend toward the highest increase in protease activity was
332 registered after the combined treatment.
333
Lysozyme-like activity
335 At 12 h pt, we recorded a decrease in lysozyme-like activity under the influence of both fungal
336 infection and avermectins (effect of fungus: $H_{1.56}$ = 6.46 p = 0.011; effect of avermectins: $H_{1.56}$ =

337   14.04 p = 0.00017; Fig. 7). The greatest decrease was observed after the combined treatment
338   (Dunn's test: P < 0.001 compared with the other treatments). At 48 h pt, a sharp (1.7–2.0-fold)
339   increase in lysozyme-like activity was recorded under the influence of avermectins ($H_{1.116}$ =
340   68.6, P < 0.0000001). The effect of the fungus on the level of the enzyme at 48 h pt was not
341   significant. No significant interaction effect between the factors on the level of lysozyme was
342   observed at 12 and 48 h pt (H = 0.23, P = 0.63).
343
344   **Fungal and bacterial CFUs**
345   The plating of mosquito larval homogenates on modified Sabouraud agar showed significant
346   differences in the Metarhizium CFUs between treatment with the fungus either alone or
347   combined with avermectins (Fig. 8A). The Metarhizium CFU count in the fungal treatment was
348   twice as high as that in the combined treatment (t = 6.4, df = 18, P = 0.001). Homogenates of
349   larvae from the fungus-free treatments (avermectin alone and control) did not form any fungal
350   colonies.
351   The plating of larval homogenates on blood agar showed a significant (17–75-fold) increase in
352   bacterial CFUs after treatment with the fungus and avermectins. A significant effect was
353   registered for avermectins ($H_{1.19}$ = 4.8, P = 0.03; Fig. 8B) but not for the fungus ($H_{1.19}$ = 1.9, P =
354   0.17). However,a clear tendency toward an increase in CFUs was registered after treatment with
355   the fungus alone (Dunn's test, P = 0.054 compared to control). No significant interaction effect
356   between the fungus and avermectins on bacterial CFUs was revealed ($H_{1.19}$ = 1.9, P = 0.17).
357
358   **Discussion**
359   We showed a synergistic effect between avermectins and Metarhizium fungi on aquatic
360   invertebrates for the first time. A similar effect was shown previously only in terrestrial insects
361   (Colorado potato beetle, cotton moth) (*Anderson et al., 1989; Asi et al., 2010; Tomilova et al.,*
362   *2016*), which are characterized by a completely different mode of fungal penetration (through the
363   exo-skeleton). The accumulation of conidia of the fungus mainly in the gut lumen of mosquitoes
364   coincides with studies of other researchers (*Butt et al., 2013*). However, we report the first
365   observation of systemic colonization of *Ae. aegypti* larvae after inoculation with Metarhizium
366   conidia. It was previously suggested that only blastospores (and not conidia) are able to
367   germinate from the gut lumen into the hemocoel of mosquito larvae (*Alkhaibari et al., 2016,*
368   *2018*). Interestingly, the larvae treated with avermectins accumulated a lower amount of conidia,
369   but this dose was sufficient for a synergistic effect on mortality. Apparently, the reduced
370   accumulation of conidia was due to the specific binding of avermectins in the bodies of
371   invertebrates with glutamate-dependent chlorine channels / receptors, which are located mostly
372   in the swallowing and somatic muscles (*Etter et al., 1999; Forrester et al., 1999*). As a result, the
373   larva's ability to swallow food, including the conidia of the fungus, is blocked.
374   We observed a decrease in PO activity and dopamine levels under the influence of the fungus,
375   whereas in terrestrial arthropods, these enzymes are activated during mycoses (*Ling & Yu, 2005;*
376   *Yassine, Kamareddine & Osta, 2012; Yaroslavtseva et al., 2017; Chertkova et al., 2018*). It has
377   been suggested that DA release is associated with the general stress reactions related to the
378   insect's responses to pathogens (*Hirashima, Sukhanova & Rauschenbach, 2000; Chertkova et*
379   *al., 2018*). In addition, DA is involved in the modulation of energetic metabolism and general
380   defense mechanisms such as phagocytosis (*Wu et al., 2015*). PO is involved in the inactivation of
381   fungal propagules in the cuticle and hemocoel (*Butt et al., 2016*). Dopamines are involved in the
382   PO cascade (*Andersen, 2010*); however, synchronous and unidirectional changes in the levels of

383   PO and dopamines are not always observed during infections (Chertkova, 2016, unpublished
384   data).
385   Perhaps conidia entering the intestine of *Ae. aegypti* larvae are able to inhibit the initiation of the
386   PO cascade. Interestingly, Alkhaibar et al. (*2018*) noted a short-term increase in PO activity in
387   whole-body homogenates of *Culex quinquefasciatus* larvae after infection with conidia or
388   blastospores of *M. brunneum* (4–6 h pt). It is possible that this reaction depends on the type and
389   strain of the pathogen as well as the type and line of mosquitoes. We observed activation of
390   detoxifying enzymes (GST, EST) in *Ae. aegypti* larvae at the early stages of toxicosis and
391   infection (12 h pt) under mono-treatments with the fungus and avermectins. However, the
392   combined treatment led to inhibition of the activation of GST and EST. A similar effect was
393   observed at 12 h pt for antibacterial (lysozyme-like) and acid protease activities. Combined
394   treatment leads to either inhibition or containment of the activation of these enzymes. GST and
395   EST are used by insects to inactivate toxic products formed by insecticide-induced toxicosis
396   (*DeSilva et al., 1997; Boyer et al., 2006; Aponte et al., 2013*) as well as under mycosis (*Serebrov*
397   *et al., 2006; Han et al., 2013*) by reducing oxidative stress and speeding up the metabolism of
398   toxins. Lysozyme inhibits the reproduction of Gram-positive bacteria (*Abdou et al., 2007;*
399   *Gandhe et al., 2007; Chapelle et al., 2009*), which (e.g., Microbacteriaceae) are among the
400   dominant bacteria in *Ae. aegypti* larvae (*Kerri et al., 2014*). It should also be noted that at 12 h pt
401   of *Ae. aegypti* with blastospores of *M. brunneum*, a decrease in the expression levels of genes
402   encoding defensins and cecropins (*Alkhaibari et al., 2016*), which inhibit the growth of both
403   Gram-positive and Gram-negative bacteria and fungi, was observed (*Jozefiak et al., 2017*). The
404   inhibition of acid protease activity under combined treatment may indicate disorders in food
405   consumption and absorption. Disruption of food absorption and starvation can increase mortality
406   from both fungi and insecticides (*Furlong, Groden, 2003*). Thus, we assume that the
407   physiological causes of the observed synergism lie in the initial stages of the development of
408   infection and toxicosis. In the later stages (48 h pt), we mainly observed activation of enzymes
409   (PO, GST, EST, acid proteases and lysozyme-like activity), which apparently indicates
410   destructive processes in tissues and organs under the action of both avermectins and fungi. The
411   increase in PO activity on the second day after treatment with avermectins was probably due to
412   the destruction of hemocytes and the release of intracellular proPO components. We have
413   previously shown the cytotoxic effect of avermectins on hemocytes, leading to their death
414   (*Tomilova et al., 2016*). Additionally, the cytostatic and cytotoxic effects of the avermectins
415   complex on various cells of warm-blooded animals are well known (*Sivkov et al., 1998; Kokoz et*
416   *al., 1999; Korystov et al., 1999; Maioli et al., 2013*). It should also be noted that an increase in
417   PO in insects can be observed with the development of various bacterioses (e.g., *Grizanova et*
418   *al., 2014; Yaroslavtseva et al., 2017*), which is consistent with the observed increase in CFUs
419   under the influence of avermectins. An increase in GST under mycoses usually correlates with
420   the severity of the infectious process (*Dubovskiy et al., 2012; Tomilova et al., 201*9) and
421   confirms the results obtained by Butt et al. (*2013*) when studying the pathogenesis of *M.*
422   *brunneum* in *Ae. aegypti* larvae. An increase in lysozyme-like activity under the action of
423   avermectins could have occurred due to tissue destruction accompanied by the release of
424   lysosome contents containing lysozyme (*Zachary, Hoffmann, 1984*). The activation of proteases
425   at 48 h pt under the influence of avermectins is correlated with the increase in PO and lysozyme-
426   like activity, which also indicates destructive changes in the tissues.
427   We observed an increase in the number of cultivated bacteria in the larvae when treated with
428   both avermectins and fungi. This effect may be associated with impaired intestinal peristalsis as

429 well as changes in the level of PO and antibacterial activity in the initial stages of toxicosis and
430 fungal infection. Similar effects have been observed in terrestrial insects following topical
431 infection by fungi (*Wei et al., 2017; Ramirez et al., 2018; Polenogova et al., 2019*) and are
432 associated with the redistribution of immune responses between the cuticle and the intestines. In
433 mosquito larvae, the fungus comes into direct contact with intestinal bacteria, which may exhibit
434 fungistatic properties (*Sivakumar et al., 2017; Zhang et al., 2017*, etc.) or, alternatively, may act
435 as synergists of fungi, as shown by Wei et al. (*2017*) in the imagoes of the mosquito *Anopheles*
436 *stephensi*. It is possible that conflicting data on the systemic colonization of mosquito larvae by
437 Metarhizium fungi are associated with differences in bacterial communities, which requires
438 further research. In any case, fungi cannot successfully completely colonize an aquatic
439 environment, and bacterial decomposition is observed in mosquito larvae, whereas fungus
440 germination occurs in the air environment.
441
442 **Conclusions**
443 In conclusion, this is the first study of the survival and physiological reactions of mosquito larvae
444 under the combined action of avermectins and entomopathogenic fungi. The synergism observed
445 under the combined action of these agents appears to be associated with physiological changes in
446 the early stages of toxicosis and infection. In particular, inhibition of the activity of a number of
447 enzymes is observed under the combined treatment associated with the detoxifying and immune
448 systems. Systemic colonization of mosquito larvae by the fungus Metarhizium is shown for the
449 first time in this study. Further investigations may be focused on studying the role of
450 endosymbiotic mosquito bacteria in the development of toxicosis and mycoses as well as the
451 development of preparative forms based on fungi and avermectins for regulating the number of
452 mosquitoes in natural conditions.
453

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457

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Mortality dynamics of *Ae. aegypti* larvae after treatment with *M. robertsii* (1×10^6 conidia/ml), avermectins (0.00001%) and their combination.

The control was treated with distilled water. The asterisks (*) indicate a synergistic effect (χ^2 > 18.5, df = 1, P < 0.001, see Table S1).

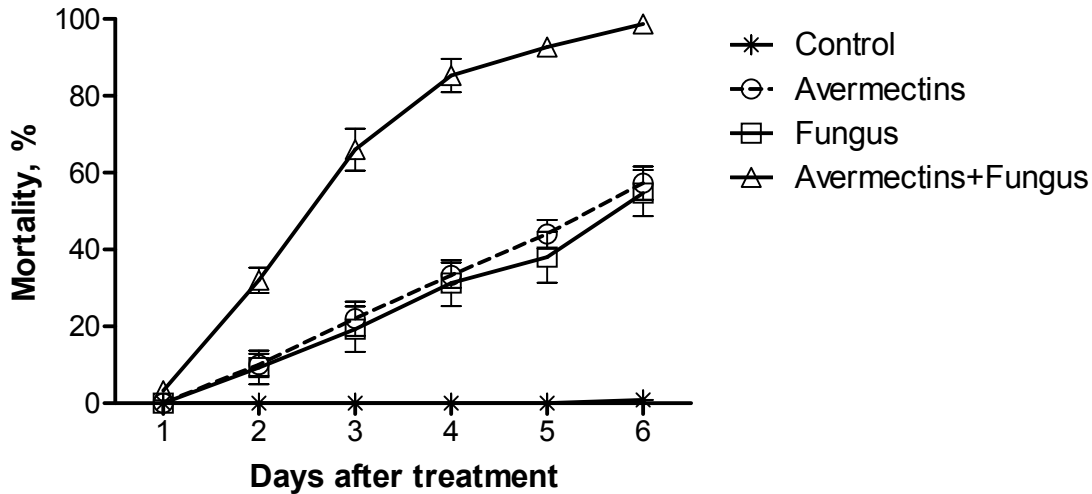
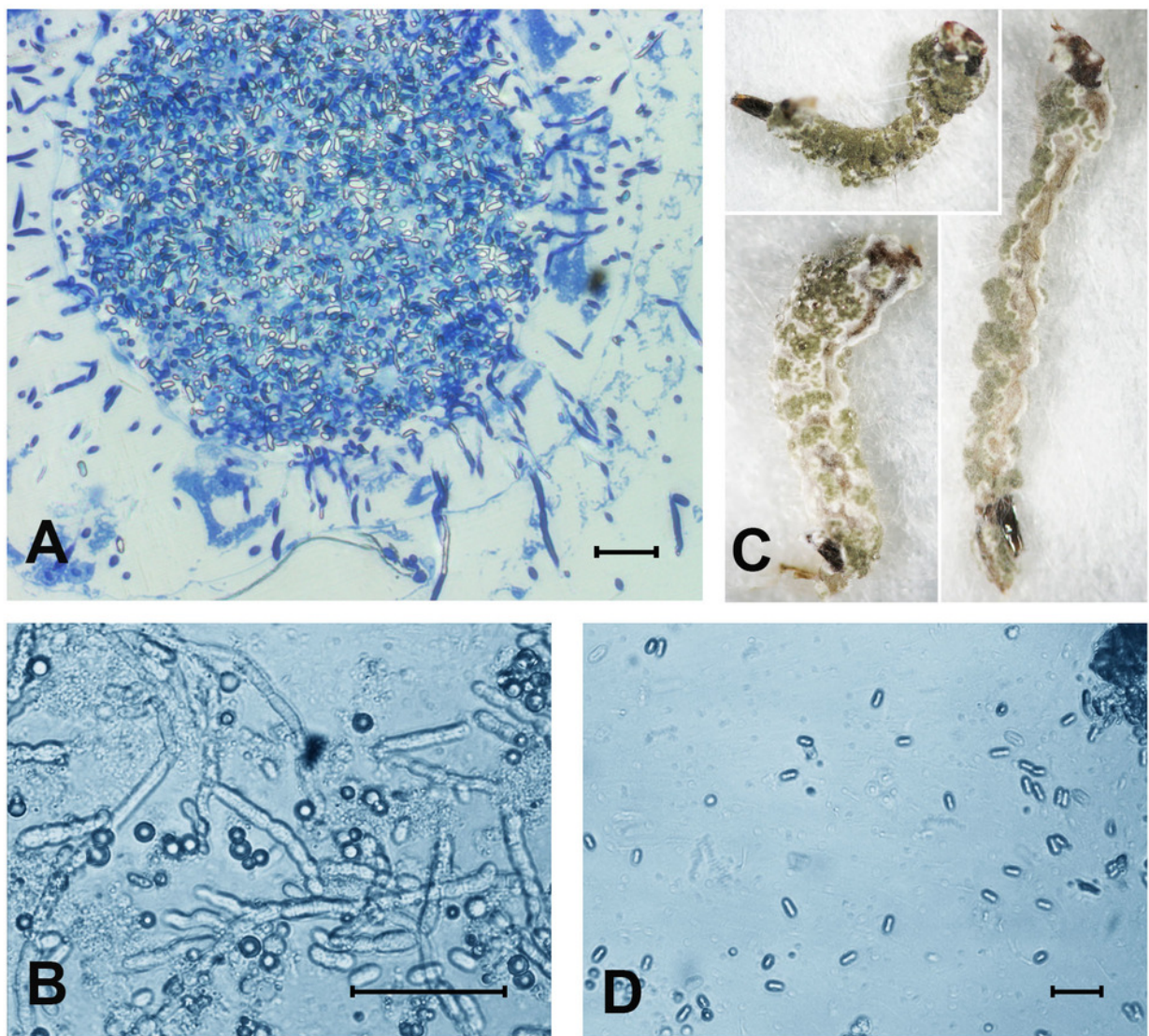
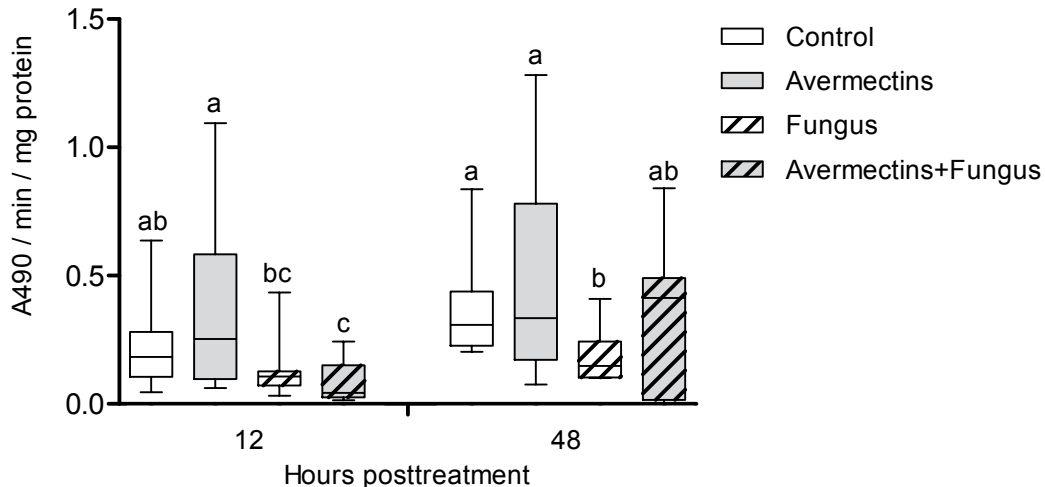
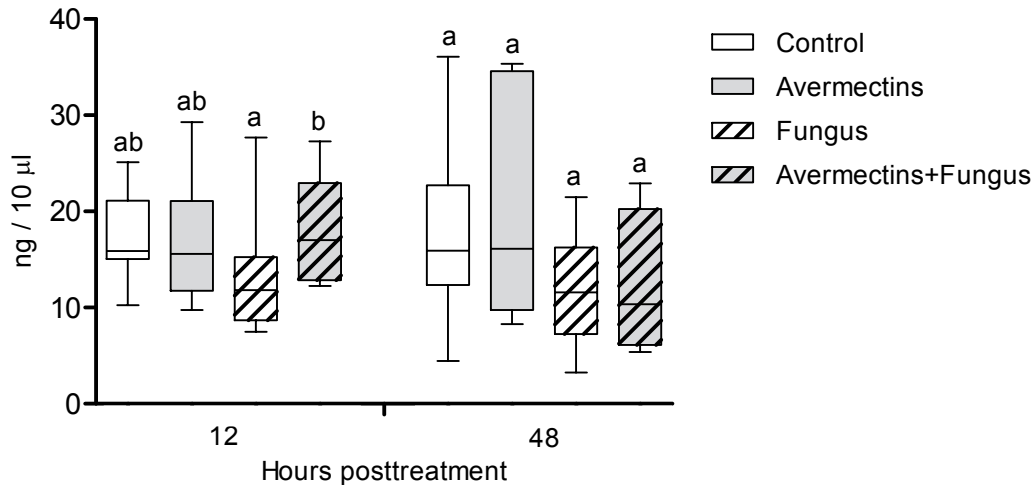



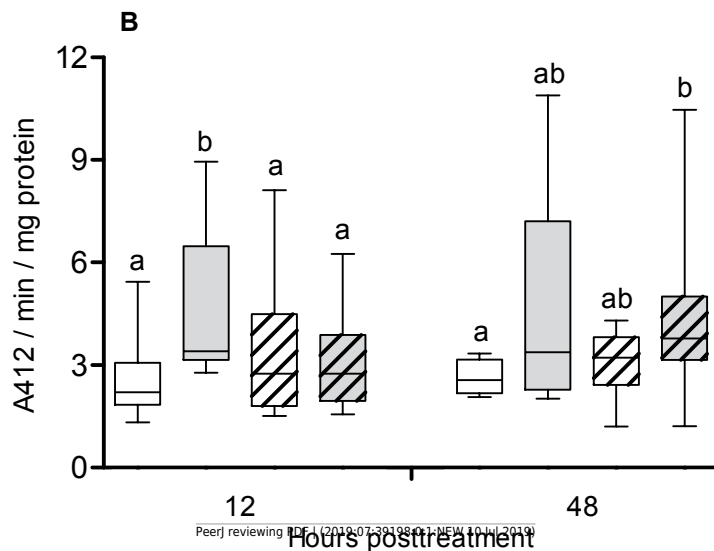
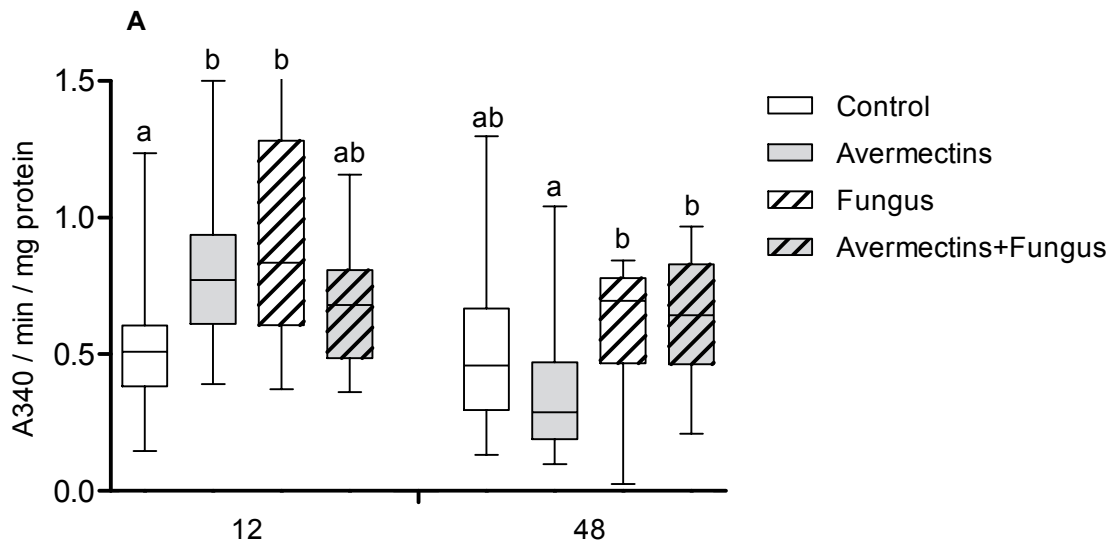
Figure 2

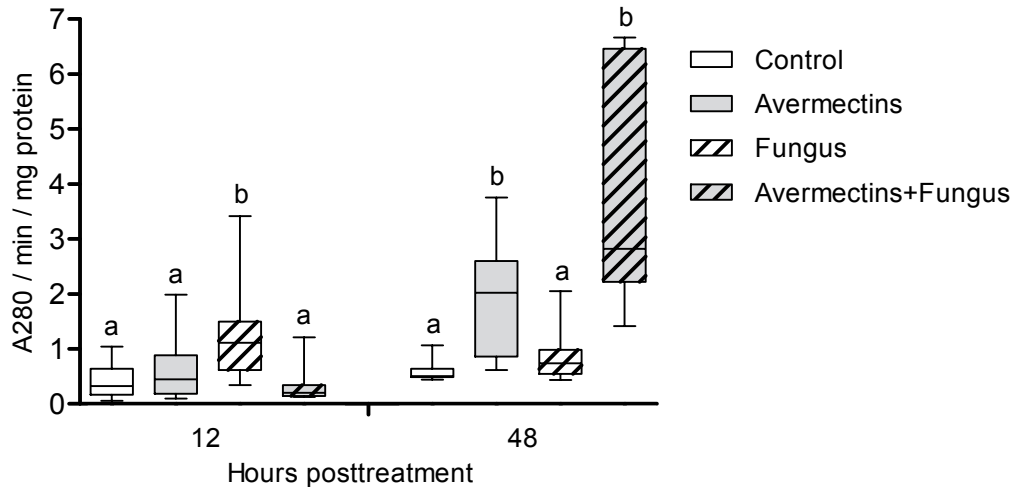
The colonization of *Ae. aegypti* by Metarhizium

A – accumulation of conidia in the intestine and colonization of the hemocoel by hyphal bodies; B – colonization of the fat body; C – mosquito larvae with germinated Metarhizium conidia in a dampening chamber; D – nongerminated conidia in a sample of water in which mosquito larvae were maintained. Scale bar: 20 μm.

Activity of PO in the whole-body homogenates of *Ae. aegypti* larvae after treatment with *M. robertsii*, avermectins and their combination.

In the control treatment, equal amounts of water were added. Boxes denote interquartile ranges (25–75%) bisected horizontally by median values; whiskers indicate min and max values. Significant differences are indicated with different letters within a time point.

Dopamine concentration in whole-body homogenates of *Ae. aegypti* larvae after treatment with *M. robertsii*, avermectins and their combination.In the control treatment, equal amounts of water were added.

Boxes denote interquartile ranges (25–75%) bisected horizontally by median values; whiskers show min and max values. Significant differences are indicated by different letters within a time point.

GST (A) and EST (B) activity in whole-body homogenates of *Ae. aegypti* larvae after treatment with *M. robertsii*, avermectins and their combination.In the control treatment, equal amounts of water were added.

Boxes denote interquartile ranges (25–75%) bisected horizontally by median values; whiskers show min and max values. Significant differences are indicated by different letters within a time point.

Acid protease activity in whole-body homogenates of *Ae. aegypti* larvae after treatment with *M. robertsii*, avermectins and their combination.In the control treatment, equal amounts of water were added.

Boxes denote interquartile ranges (25–75%) bisected horizontally by median values; whiskers show min and max values. Significant differences are indicated by different letters within a time point.

Lysozyme-like activity in whole-body homogenates of *Ae. aegypti* larvae after treatment with *M. robertsii*, avermectins and their combination.In the control treatment, equal amounts of water were added.

Boxes denote interquartile ranges (25–75%) bisected horizontally by median values; whiskers show min and max values. Significant differences are indicated by different letters within a time point.

Colony forming units of *M. robertsii* (A) and cultivable bacteria (B) in whole-body homogenates of *Ae. aegypti* larvae after treatment with *M. robertsii*, avermectins and their combination.

Boxes denote interquartile ranges (25 – 75 %) bisected horizontally by median values.

Whiskers show min and max values. Significant differences are indicated by different letters.