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ORIGINAL ARTICLE

New insights into the infection of the American cockroach *Periplaneta americana* nymphs with *Metarhizium anisopliae* s.l. (Ascomycota: Hypocreales)

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behaviour, cockroach, cuticular fatty acids, entomopathogenic fungus, haemolymph.

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Abstract

Aims: To study the marked resistance of *Periplaneta americana* to entomopathogenic *Metarhizium anisopliae*.
Methods and Results: The low susceptibility of 4th instar nymphs applied topically with conidia seemed to be related to an active removal of conidia by the cockroach and to a disabled or retarded germination and subsequent development of conidia on the cuticle (up to 80% germination in the next 7 days after application). Inhibitions or delays of germination were related to the composition of the epicuticular fatty acids (30·1% w/w oleic, 28·3% w/w linoleic, 24·5% w/w palmitic and 11·7% w/w stearic acid) reported here. Propagules invading the nymphs through the cuticle took at least 3 days to reach the haemocoel, and no propagules were found after day 8 post-treatment. Strain IP 46 infected >50% of nymphs treated with doses $\geq 2 \times 10^4$ hyphal bodies (HB) nymph^{-1} and reduced the survival of nymphs ≤50%. Most nymphs (>70%) survived after injection of 6×10^3 and 2×10^3 HB nymph^{-1}.
Conclusions: Findings emphasize a distinct resistance of nymphs of the American cockroach to infections by *M. anisopliae*.
Significance and Impact of the Study: Our findings provide support for the development of biological control of this synanthropic cockroach pest.

Introduction

In the tropics and subtropics *Periplaneta americana* (Blattodea: Blattidae) is one of the most common synanthropic insect pests in domestic residences, restaurants, hospitals and other human facilities. This cockroach occurs in both out- and indoor areas and easily proliferates in subterranean utility network tunnels, especially sewer systems, from which this pest invades the mentioned facilities (Jones 2008). The species emits an obnoxious odour and contaminates human environments with faeces and debris of exuviae, remains of oothecae and dead individuals. In addition, *P. americana*, like other domestic cockroaches, can cause allergies and entomophobia (Nowak-Wegrzyn *et al.* 2009; Mindykowski *et al.* 2010), and acts as a mechanical vector of pathogens and parasites (Pai *et al.* 2005; Lemos *et al.* 2006).

Cockroaches are mostly controlled by low-cost, relatively fast-acting chemical insecticides (Cochran 1999; Rahman and Akter 2006). However, indiscriminate use of chemicals is a major reason for the increasing resistance of these pests (Karunaratrie 1999; Qian *et al.* 2010), and there is therefore an urgent need to search for innovative control methods. Efforts to control cockroaches in recent years have also included entomopathogenic fungi, all of which invade their hosts through the cuticle. Fungi were evaluated particularly against *Blattella germanica* (Blattodea: Blatellidae) with promising results (Quesada-Moraga *et al.* 2004; Lopes and Alves 2011), and interest in *P. americana* as a target pest for biological control with

mycoinsecticides has increased (Murali *et al.* 1999; Hubner-Campos *et al.* 2013; Mutyala and Vadlamani 2014). The resistance of this cockroach to fungal infection is still poorly understood and may be connected eventually to inherent behavioural, mechanical and physiological defence mechanisms. There are only few reports on the natural occurrence of entomopathogenic fungi in synanthropic or sylvatic cockroaches (Roth and Willis 1960; Cornwell 1968; Montalva *et al.* 2016).

The insect cuticle is the first host barrier against infections by fungal pathogens. Adhesion and germination of infective conidia on the cuticle and subsequent penetration of hyphae can be reduced or even interrupted by antifungal compounds in the cuticle, and cellular and humoral defence reactions will also determine the insect's susceptibility or resistance to fungal invasion and infection (Gunnarsson and Lackie 1985; Vilçinskas and Götz 1999). Compounds in the epicuticle affect both conidial germination and hyphal growth, and are related to specific susceptibility patterns of the host (Boucias and Latgé 1988; Sosa-Gomez *et al.* 1997; Wang and St. Leger 2005; Pedrini *et al.* 2007; Gołębiowski *et al.* 2011). The specific chemical compositions of the epi- and procuticle probably exert an important role in the pathogen-host recognition and host defence mechanisms during fungal penetration (Koidsumi 1957; Gołębiowski *et al.* 2011). We investigated the cuticular lipids of *P. americana* and present new findings on the activity of *M. anisopliae* s.l. IP 46, active against this cockroach (Hubner-Campos *et al.* 2013) but also against other insects such as triatomines (Rocha and Luz 2011; Luz *et al.* 2012; Rodrigues *et al.* 2015) and mosquitoes (Mnyone *et al.* 2009; Santos *et al.* 2009; Sousa *et al.* 2013; Lobo *et al.* 2016), in this cockroach and some factors that affect the infection process.

Material and methods

Rearing of cockroaches

Periplaneta americana individuals used for the bioassays were reared at the Instituto de Patologia Tropical e Saúde Pública (IPTSP), Universidade Federal de Goiás, Brazil. The colony originated from individuals collected in 2009 in Goiânia, Goiás, Brazil. Cockroaches were held in plastic boxes with lids (38 × 27 × 24 cm) provided with cardboard shelters. They were fed with commercial dry dog-food (Purina Dog Chow®; Nestlé, São Paulo, Brazil), were offered tap water *ad libitum* through moistened cotton arranged in a plastic cup filled with water (200 ml) and were maintained at 25 ± 3°C, relative humidity (RH) of 75 ± 10% and natural photophase (Hubner-Campos *et al.* 2013). Reported assays with cockroaches needed no approval by the Ethics Commission for the Use of Animals, UFG.

Extraction of cuticular lipids

Five 4th instar nymphs (N4) and adults of *P. americana* were tested separately to determine fatty acid from the cuticle. Cockroaches were euthanized with CO_2, and then the cuticle dissected under a stereomicroscope (Zeiss Stemi DV4, Carl Zeiss, Inc., Oberkochen, Germany) in a 0·85% physiological NaCl solution. Fat body residues were removed from the cuticle with cotton previously wetted with the same salt solution and then discarded. The processing of samples consisted of total extraction of cuticular lipids with a Folch mix (chloroform/methanol 2 : 1 v/v) in a ratio of 20 : 1 w/v relative to the sample mass (Folch *et al.* 1957). A partition was performed with 20% v/v distilled water. The upper methanolic phase was discarded, and the remaining chloroformic phase evaporated to dryness under a nitrogen stream. The dry extract was then saponified with a 10% solution of potassium hydroxide in methanol for 1 h at 80°C to remove sterols, pigments and other unsaponifiable lipids whose presence interferes with the analysis of the fatty acids. The unsaponifiable material was extracted with petroleum ether for 5 min. Potassium soaps of fatty acids present in the samples were then acidified with concentrated hydrochloric acid, a process that releases fatty acids. Free fatty acids were treated with 10% boron trifluoride in methanol, in a nitrogen atmosphere and an electric hot plate for 30 min at 80°C. This procedure converts fatty acids in volatile methyl esters derivatives, a necessary condition for the subsequent analysis by gas chromatography. The methyl esters were extracted with petroleum ether for 5 min. The composition of fatty acids was determined by gas chromatography using a capillary column with 50 m length, 0·25 mm internal diameter and 0·1 μm film thickness (Chrompack CP SIL 88, Santa Clara, CA, USA) on a gas–liquid chromatograph (Hewlett Packard 6890) equipped with a Flame Ionization Detector (FID). The analytical conditions were 185°C initial temperature for 3 min, heating ramp rate of 3°C per minute up to 230°C, then maintaining 230°C for 25 min. The FID temperature was set at 280°C. The fatty acid profile was obtained by comparing the relative retention times with commercial standards (Nu-Check Prep, Inc., Elysian, MN) analysed previously in the same column.

Origin, culture and preparation of inoculum of conidia and hyphal bodies

Metarhizium anisopliae s.l. IP 46 was isolated in 2001 from soil collected in the Ema National State of Goiás, Brazil (Rocha *et al.* 2012). This strain was also deposited as CG 620 in the Fungal Culture Collection of Embrapa Recursos Genéticos e Biotecnologia, Brasília, Brazil. The fungus was cultured on Potato-Dextrose-Agar medium

(PDA; Difco, Sparks, MD) at 25 ± 1°C and 12 h photophase. Conidia were scraped from a 7-day old culture, with a sterile spatula, and suspended in 5 ml of 0·01% (v/v) Tween 80 (polyoxyethylene sorbitan monoleate) in a sterile plastic tube of 45 cm^3. The suspension was vortexed for 3 min, filtered through hydrophilic cotton, then the number of conidia was quantified with a Neubauer chamber, and final conidial concentrations adjusted. The viability (>98%) of conidia and hyphal bodies (HB) used in the treatments was confirmed as described by Lane *et al.* (1988). Fungal inoculum for topical application (at 10^9 conidia ml^{-1}) was prepared as oil-in-water formulation at 10% emulsifiable vegetable oil (Graxol®; Agrária Indústria e Comércio Ltda, Jardinópolis, SP, Brazil), and the same emulsion without conidia for the controls.

HB for injection were produced in modified Adamek′s-Corn medium: 3 ml corn steep liquor, 4 g yeast extract, 4 g glucose, 2 ml Tween 80® at 0·1% and 95 ml sterile water (Adamek 1963). The culture medium (100 ml) in an Erlenmeyer flask (250 ml) was inoculated with conidia at a final concentration of 10^4 conidia ml^{-1} and incubated at 28 ± 1°C and 150 rev min^{-1} for 4 days. The medium was then filtered through a sterile muslin layer, and the filtrate centrifuged at 43·546 RCF (relative centrifugal force) for 20 min. The pellet with the HB was suspended in 20 ml of sterile water, centrifuged as mentioned and finally resuspended in sterile 0·85% NaCl solution. HB were quantified as described for conidia, and concentrations adjusted to 10^7, 3×10^6, 10^6, 3×10^5 and 10^5 HB ml^{-1} of 0·85% saline.

Topical application and injection of fungal inoculum and further procedures

Nymphs were sedated by a 30 s exposure to CO_2 or until complete sedation. For the topical inoculation, 15 μl of the inoculum were applied on the dorsal area of both thorax and abdomen of each nymph with a semi-automatic pipette at a final dose of $1{\cdot}5 \times 10^7$ conidia $nymph^{-1}$. Control groups were treated equally with the emulsion only. Immediately after topical application, nymphs were placed on sterile filter paper into a Petri dish (160 × 25 mm) and incubated at 25 ± 1°C, >98% RH, and 12 h photophase for up to 10 days. Their behaviour was examined within an hour of inoculation and then daily before retrieving living or dead individuals. For this behavioural check, the mobility of nymphs was confirmed by tapping lightly on the lid of the Petri dish. Ecdysis of nymphs was assessed daily, and any exuviae removed from the dishes. Dry dog-food and water were placed inside the Petri dishes as noted above and changed every 3 days.

Mortality was checked daily; dead nymphs were transferred onto sterile filter paper in Petri dishes (90 × 15 mm) and incubated in a humid chamber at 25 ± 1°C to allow fungal development. Individuals with evidence of external fungal growth were examined with a stereomicroscope (Motic SMZ-143; 40–80 X), and fungal microstructures were examined with a brightfield microscope (Leica DMLS 020-518.500; 400–800 X, Leica Microsystems, São Paulo, Brazil) to confirm the fungal species.

For each repetition, a total of 30 nymphs was used with 5 N4 in each dish. The test was repeated four times on different dates.

For parenteral inoculation, groups of nymphs were immobilized as mentioned previously, and 20 μl of the HB suspension injected ventro-laterally into the haemocoel through the first abdominal segment with a 1 ml disposable syringe (13 × 0·45 mm, 100 U.I., Top Med®, Juiz de Fora, MG, Brazil) at a final dose of 2×10^5, 6×10^4, 2×10^4, 6×10^3 or 2×10^3 HB. Control nymphs were injected 20 μl sterile 0·85% saline. Nymphs were then held for up to 10 days at 25 ± 1°C, 75 ± 10% RH, and 12 h photophase. A total of 20 N4 was injected for each dose, and the test was repeated four times.

Haemolymph samples were obtained daily from live individuals previously treated for up to 10 days by topical application or injection with conidia or HB respectively. For this process, each nymph was surface-sterilized by completely dipping in 70% ethanol for 3 s and subsequently rinsed twice in sterile water for 3 s. One of the metathoracic legs was then cut at the base with fine scissors, and 20 μl of haemolymph were collected with a micropipette. Ten microlitres of the haemolymph were transferred to an Eppendorf tube (1·5 ml) with 190 μl sterile 0·85% saline and vortexed for 10 s. The number of HB μl^{-1} haemolymph was determined daily with a Neubauer chamber for up to 10 days after treatment.

The number of Colony Forming Units (CFU) μl^{-1} haemolymph on culture medium was determined with the remaining 10 μl of haemolymph after a 10-fold dilution with 0·85% saline. The mixture was spread with a Drigalsky spatula onto Sabouraud-Dextrose-Yeast Agar medium (quarter strength SDYA; 1% peptone, 0·25% dextrose, 0·2% yeast extract, and 1·5% agar) supplemented with chloramphenicol (0·05%) in a Petri dish (90 × 15 mm) and then incubated at 25 ± 1°C, 75 ± 10% RH and 12 h photophase. The development and number of CFU on the medium were assessed in the next days. The survival and fungal infection of nymphs were monitored daily up to 10 days.

Preparation of samples for scanning microscopy

N4 were set individually on sterile filter paper in a Petri dish (160 × 25 mm) and sedated as mentioned. Oil-in-water formulated conidia (15 μl with a total $1{\cdot}5 \times 10^7$

conidia) were applied topically as mentioned. Control nymphs were treated in the same way with oil-in-water emulsion only. All nymphs were maintained up to 168 h at 25 ± 1°C, >98% RH and 12 h photophase and supplied with food and water as mentioned.

At 20 min, 24 h, 48 h, 72 h and 168 h after inoculation nymphs were euthanized with CO_2 and processed. Antennae of nymphs were removed with scissors after 20 min exposure and fixed overnight at 4°C in (2% paraformaldehyde, 2% glutaraldehyde in 0·1 mol l^{-1} sodium cacodylate buffer, pH 7·2). After a 24, 48, 72 or 168 h exposure the dorsal sections of the thorax and abdomen were removed and fixed as mentioned. All samples were dehydrated after fixation in a series of ethanol solutions (30, 50, 70, 90 and 100% for 15 min each, repeating 100% once for another 15 min). The dehydrated samples were critical-point dried (Autosamdri-815; Tousimis Research Corp, Rockville, MD), sputter-coated with gold and finally examined with a scanning electron microscope (JSM 6610; JEOL INC, Pleasanton, CA). Germination of conidia in a specific area of the cuticle was determined on at least 100 conidia based on four different individuals.

Analysis of data

Per cent mortalities (arcsine-square root transformed) and the number of HB and CFU were analysed with analysis of variance and the Student-Newman-Keuls multiple range test for comparison of means. Means were considered to be statistically different at $P < 0{\cdot}05$ (Statistica, Tulsa, OK, USA). The survival of nymphs was analysed by Kaplan–Meier method, and the log rank test was used to test whether there is a difference between the survival times of different groups, using SPSS ver. 16 (SPSS Inc., Chicago, IL, USA).

Results

Cuticular fatty acids of nymphs and adults of *Periplaneta americana*

A total of 14 fatty acids was detected by gas chromatography, mostly straight- and long chained or very long chained with chains of 11–24 carbon atoms, eight of them saturated and six unsaturated, were extracted from the cuticle of nymphs and adults of cockroaches analysed. There was a marked dominance of fatty acids containing 16–18 carbon atoms (90% w/w), and the predominant fatty acids in all samples were oleic acid, linoleic acid, palmitic acid (Table 1). However, differences in the fatty acid composition and relative content of fatty acids (%) between developmental stages are presented (Table 1; Fig. 1a,b). Lignoceric acid (C24:0) was detected only in nymphs (Fig. 1a), and undecylic acid (C11:0), lauric acid (C12:0), myristic acid (C14:0), palmitoleic acid (C16:1n7), elaidic acid (C18:1n9), α-linolenic acid (C18:3n3) and arachidonic acid (C20:4n6) were detected only in adults (Table 1; Fig. 1b).

Behaviour of nymphs after topical treatment

Whether treated with oil-formulated conidia or with the emulsion only (control), active movements of antennae and forelegs on their body′s surface, especially on previously treated areas, began shortly after recovering from sedation. Antennae and legs were then brought to the mouth, drawn through the mouthparts, and this grooming procedure was repeated continuously for several minutes and to a diminishing extent over the next hours. All grooming behaviours observed were by individual insects; no mutual grooming was seen.

Within 24 h post-treatment, nymphs responded to light taps on the Petri dish lid with rapid movements for less than a minute but then their movements slowed down. Beginning 2 days after treatment, 20 up to 30% of the few fungus-treated individuals started to show clear signs of their movements becoming distinctly slower or even of complete paralysis.

Fungal development on the cuticle

High numbers of ungerminated conidia were detected on the previously treated surfaces of thorax and abdomen but also on the untreated antennae (except for the controls) (Figs 2a–c and 3a). Twenty min after application of conidia to the dorsal area of both thorax and abdomen conidia were found on antennae (Fig. 2b–c); conidial concentrations on the antennae and other treated body areas were highest in the first 24 h but then decreased in the next days (Fig. 3a–c). Small numbers of conidia, however, were found on a regular basis on the cuticle until the end of the experiment (10 days).

The germination of conidia that remained on the cuticle did not exceed 5% within the first 24 h after application but reached up to 80% in the next 7 days on individuals observed with scanning electron microscopy (Fig. 3a–c).

Fungal presence in the haemolymph after topical treatment

The majority of topically treated nymphs did not present detectable levels of HB in the haemolymph during the 10-day test period. Some individuals were found with HB (6%) in the haemolymph or CFU (8%) on culture medium after inoculation with haemolymph from treated individuals. Both HB and CFU were detected in the haemolymph only after day 3 and were not found after day 8

Table 1 Relative mean content of fatty acids (± standard error of the mean) in the cuticular lipids of nymphs and adults of *Periplaneta americana*

No	C:D*	Systematic name†	Common name	Nymphs‡	Adults‡
1	11:0	Undecanoic acid	Undecylic acid	–	0·2 ± 0·01
2	12:0	Dodecanoic acid	Lauric acid	–	0·3 ± 0·02
3	14:0	Tetradecanoic acid	Myristic acid	–	0·2 ± 0·03
4	15:0	Pentadecanoic acid	Pentadecylic acid	1·6 ± 0·1	0·7 ± 0·1
5	16:0	Hexadecanoic acid	Palmitic acid	24·5 ± 3	9·19 ± 2·2
6	16:1n7	*c* -9-Hexadecenoic acid	Palmitoleic acid	–	1 ± 0·16
7	17:0	Heptadecanoic acid	Margaric acid	1·7 ± 0·4	0·4 ± 0·03
8	18:0	Octadecanoic acid	Stearic acid	11·7 ± 0·6	8·4 ± 0·2
9	18:1n9	*c*-Octadecenoic acid	Oleic acid	30·1 ± 3·3	50·4 ± 4·9
10	18:1n9	*t* -9-Octadecenoic acid	Elaidic acid	–	1·7 ± 1·4
11	18:2n6	Octadecadienoic acid	Linoleic acid	28·3 ± 1·6	17·6 ± 0·9
12	18:3n3	*c* -9,12,15-Octadecatrienoic acid	α-Linolenic acid	–	1·2 ± 0·2
13	20:4n6	*c* -5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	–	8·71 ± 0·1
14	24:0	Tetracosanoic acid	Lignoceric acid	2·1 ± 0·3	–
				100	100

*C:D number of carbon atoms:number of double bonds.

†International Union of Pure and Applied Chemistry (IUPAC).

‡% w/w, relative content of fatty acids (%). Data are based on five separate analyses performed on different samples.

post-treatment (Fig. 4a,b). In individuals that tested positive for the presence of HB, the number of these cells varied between 5×10^2 (5 days post-treatment) and $4{\cdot}1 \times 10^7$ HB μl^{-1} haemolymph (6 days post-treatment) without significant effect of the time on the number of HB ($F_{2,40} = 0{\cdot}95$; $P = 0{\cdot}5$; Fig. 4a). The number of CFU also showed a high variability, with lowest recovery being 10 CFU and the highest recovery of $1{\cdot}6 \times 10^4$ CFU μl^{-1} haemolymph; there was no significant effect of the time after treatment on the number of CFU recovered from the treated individuals ($F_{2,40} = 0{\cdot}89$; $P = 0{\cdot}54$; Fig. 4b).

A small number of fungus-treated nymphs (20 ± 5·8%) and also of the control nymphs (15 ± 8·6%) moulted during the 10 days of the test; there was no significant effect of the treatment on the number of moulted nymphs ($F_{9,60} = 0{\cdot}64$; $P = 0{\cdot}76$; Fig. 5a). All moulted and previously fungus-treated nymphs survived until the end of the tests. The first nymphs that did not moult died 2 days after topical treatment; cumulative mortality after 10 days reached 30 ± 0·3% and showed a highly significant difference among the treatments ($F_{9,60} = 6$; $P < 0{\cdot}001$; Fig. 5b). No control mortality was observed in the same period. *Metarhizium anisopliae* developed mycelium and new conidia on half of the dead nymphs in a humid chamber.

Fungal presence in the haemolymph after injection

After injecting HB at doses between 2×10^5 and 6×10^3 HB nymph^{-1} (but not at 2×10^3 HB nymph^{-1}), HB (or CFU) were detected in the haemolymph samples after inoculating haemolymph onto culture medium only up to the 3rd days after injection (Fig. 6a,b). The HB doses applied initially had a highly significant effect on the number of HB detected in the haemolymph at the first ($F_{4,15} = 9{\cdot}18$; $P = 0{\cdot}0006$; 2×10^5 HB > remaining doses tested) and 2nd ($F_{4,13} = 10{\cdot}98$; $P = 0{\cdot}0004$; 2×10^5 HB > 6×10^4 HB and 2×10^4 HB > 2×10^3 HB), but not at the 3rd day ($F_{3,11} = 2{\cdot}52$; $P = 0{\cdot}1$). The 1st day after injection, only at the highest tested dose (2×10^5 HB), HB were detected in the haemolymph, at concentrations varying from zero to $3{\cdot}5 \times 10^4$ HB μl^{-1} among repetitions. The next day the number of HB increased to $4{\cdot}6 \times 10^5$ HB μl^{-1} in nymphs treated with this dose; HB were also detected at lower doses applied between 6×10^4 and 6×10^3 HB, but none were found after the lowest dose of 2×10^3 HB (Fig. 6a). The following day the number of HB detected in the haemolymph decreased so that at the end of the tests no HB were detected in the haemolymph regardless of the dose tested (Fig. 6a). As was found with HB detected in the haemolymph, CFU developed from haemolymph sampled between the 1st and 3rd day after injection of HB (Fig. 6b).

Metarhizium anisopliae IP 46 infected >70% of nymphs after injection of 2×10^5 and 6×10^4 HB nymph^{-1}, and these doses reduced the survival of nymphs down to 6·7 and 26·6%, respectively (Fig. 7), with median survival time (MST ± SE, 95% confidence interval) of nymphs of 3 ± 0·4 (2–3) days at 2×10^5 HB nymph^{-1} and 4 ± 0·8 (3–6) days at 6×10^4 HB nymph^{-1}. At 2×10^4 HB nymph^{-1} more than 50% of nymphs were infected, and MST ± SE (95% confidence interval) was 6 ± 0·9 (5–8) days. All nymphs treated with ≥2×10^4 HB nymph^{-1} and that subsequently died presented fungal growth on

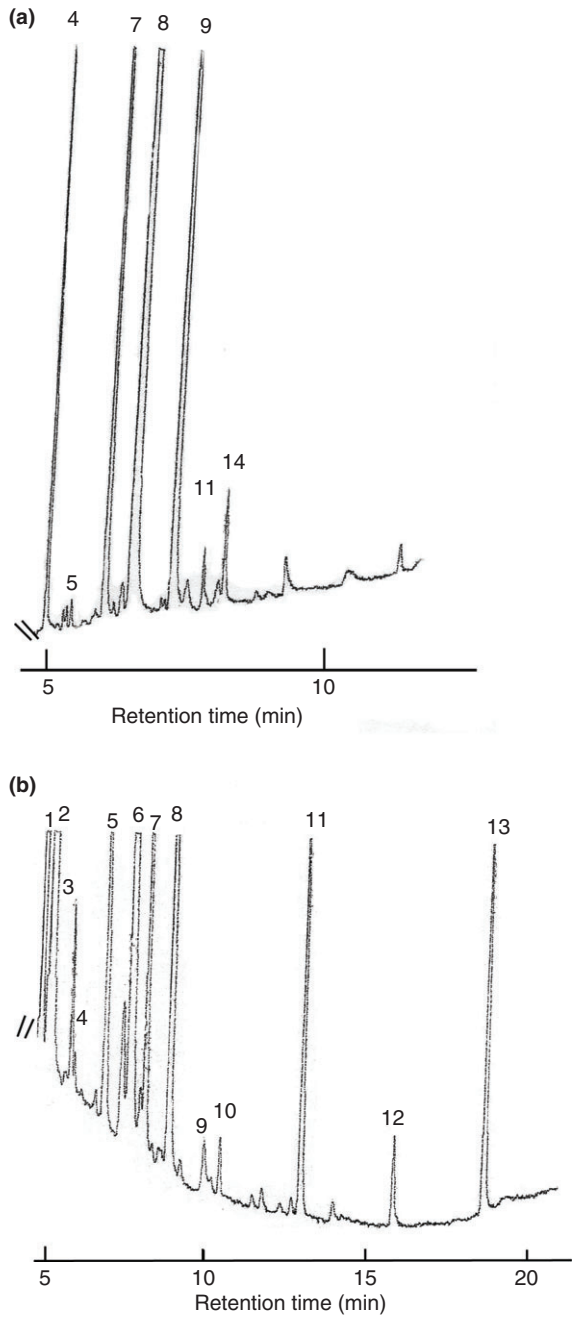

Figure 1 Capillary gas chromatographic traces of fatty acid methyl esters detected in *Periplaneta americana* cuticle of nymphs (a) and adults (b). Assigned structures are based on comparison of retention times to standard similarly runs, data base comparison of mass spectra, and on the analysis of the fragmentation patterns according to Ryhage and Stenhagen (1960). Numbers correspond to peak numbers from Table 1.

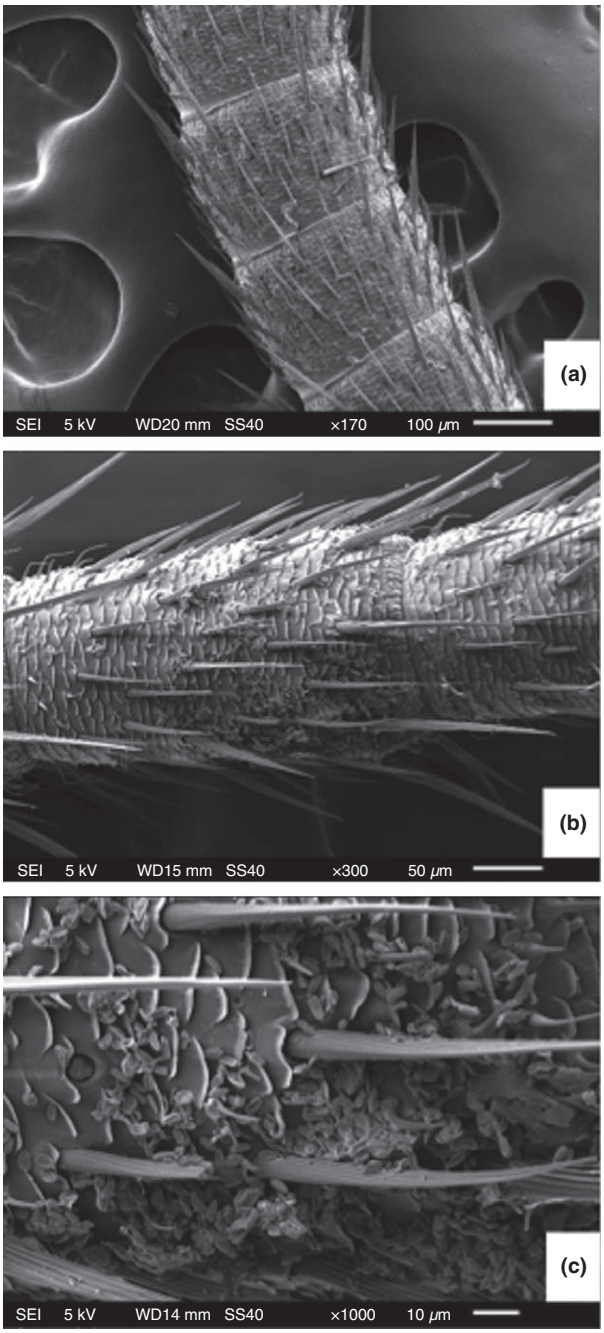

Figure 2 *Periplaneta americana* antenna of 4th instar nymph without conidia (control) (a), 20 min after application of *Metarhizium anisopliae* conidia on dorsal thorax and abdomen with presence of conidia on antenna (b, c).

their surface after incubation in a humid chamber for at least 4 days. A high number (>70%) of nymphs injected with 6×10^3 or 2×10^3 HB nymph^{-1} survived up to 10 days after treatment (Fig. 7), and the MST could not be estimated due to the low mortality. However, only 50% of the dead nymphs showed distinct fungal growth of *M. anisopliae* on their surface. In the control, >96% of nymphs were still alive after 10 days, and none of the cadavers showed external fungal development (Fig. 7). There was a highly significant effect of the dose on the survival time ($\chi^2 = 110{\cdot}5$; df = 5; $P < 0{\cdot}0001$).

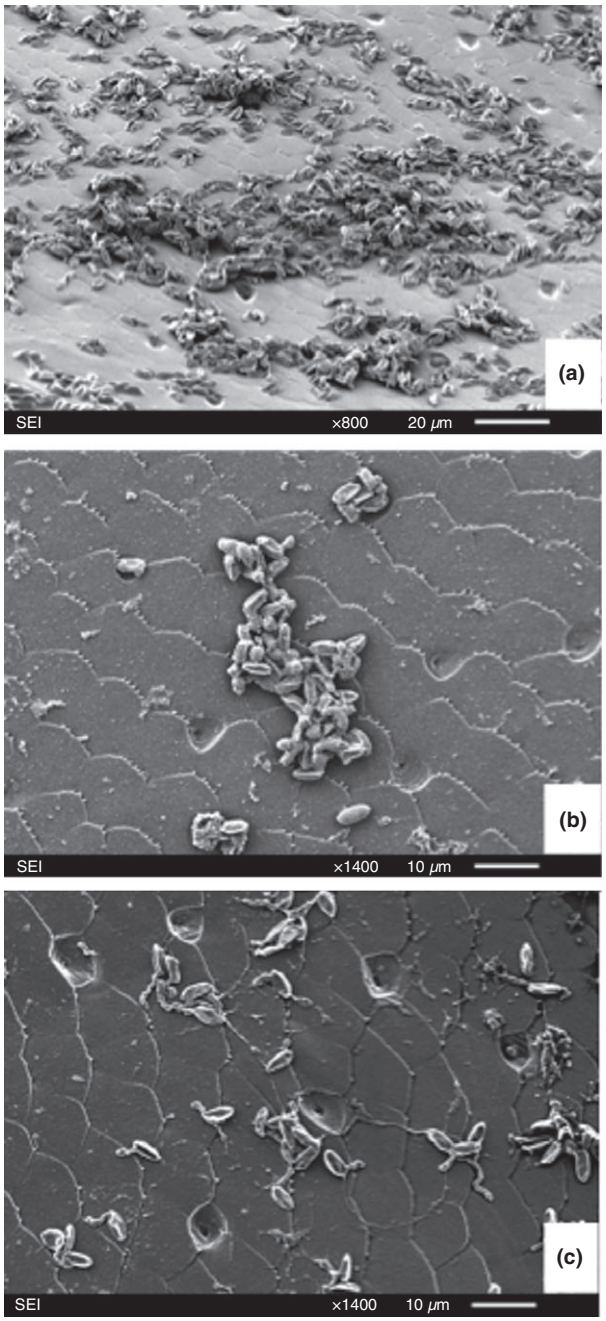

Figure 3 *Metarhizium anisopliae* conidia on *Periplaneta americana* thoracal cuticle, a large number of mostly nongerminated conidia, 24 h after application (a), reducing their number on the cuticle in the next 3 (b) and 7 (c) days and with simultaneous increasing germination.

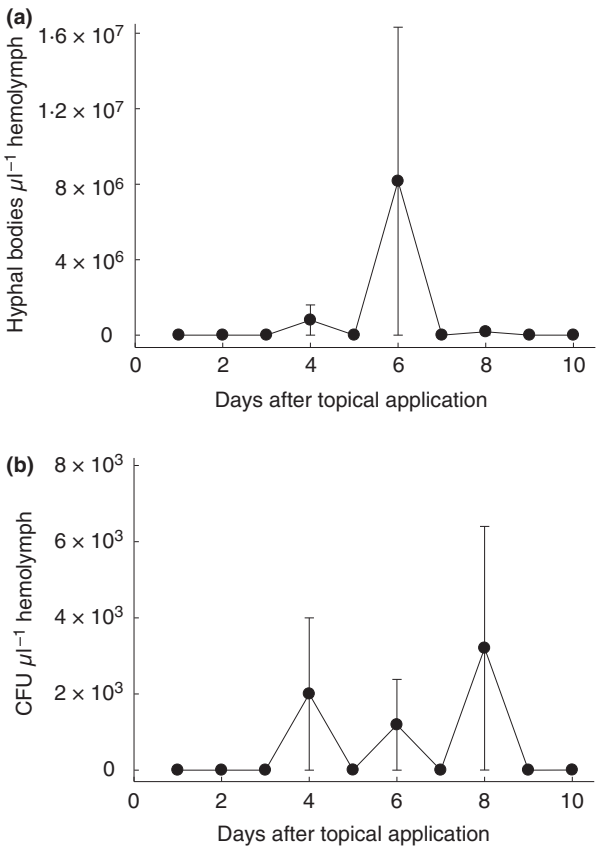

Figure 4 Mean number of hyphal bodies μl^{-1} haemolymph (± standard error of the mean, SE) (a) and mean number of Colony Forming Units (CFU) μl^{-1} haemolymph (± SE) on culture medium (b) of *Periplaneta americana* up to 10 days after topical application of *Metarhizium anisopliae* conidia ($1{\cdot}5 \times 10^7$ conidia nymph^{-1}) and incubation at 25°C and humidity close to saturation up to 10 days.

Discussion

Fungal-host insect recognition, conidial attachment to the cuticle and further fungal development are triggered by epicuticular lipids (Pedrini *et al.* 2007; Boguś *et al.* 2010). The qualitative and quantitative composition of these lipids is also critical for specific defence mechanisms against pathogens and is known to vary among host species and also among different developmental stages in the same species; the net result is observed as a stage-related susceptibility in cockroaches and other arthropods (James *et al.* 2003; Kirkland *et al.* 2004; Gołębiowski *et al.* 2011, 2013; Gutierrez *et al.* 2015). In a previous study fatty acids increased the resistance to fungal infection in important lepidopteran and muscoid pests of stored grains (Gołębiowski *et al.* 2008); the compound of fatty acids detected here in the epicuticle of *P. americana* nymphs did not prevent adhesion but eventually hampered the germination and further development of a major part of *M. anisopliae* conidia as shown previously for *Blattella germanica*, *Blaptica dubia* and *Blatta orientalis* (Gutierrez *et al.* 2015).

A large portion of conidia on the dorsal thorax and abdomen that are not easily accessible on either the antennae or forelegs were still removed by the nymphs

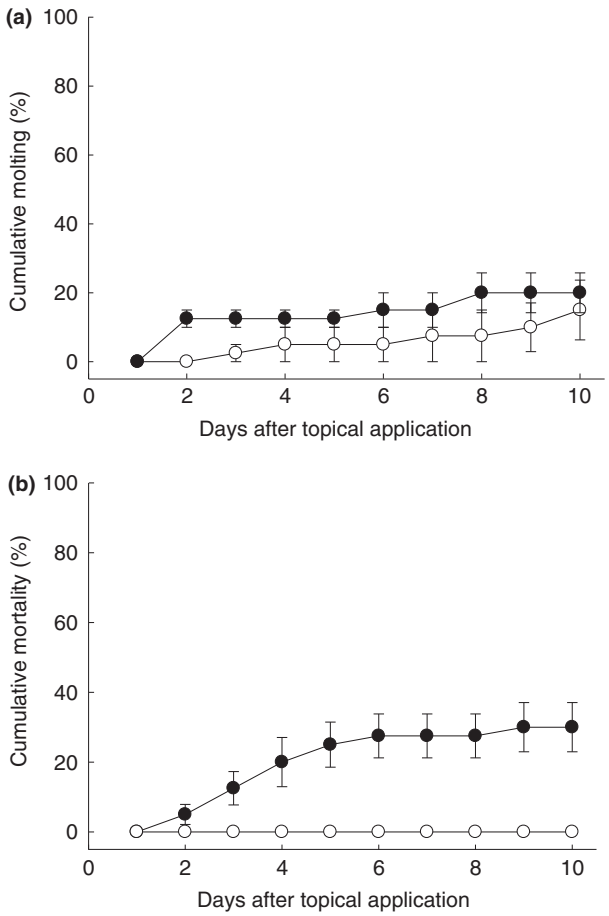

Figure 5 Cumulative relative moulting of *Periplaneta americana* (± standard error of the mean, SE) (a) and cumulative mortality (± SE) (b) after topical application of emulsion (control –○–) or *Metarhizium anisopliae* oil-in-water formulated conidia ($1{\cdot}5 \times 10^7$ conidia nymph^{-1} –●–) and incubation at 25°C and humidity close to saturation up to 10 days.

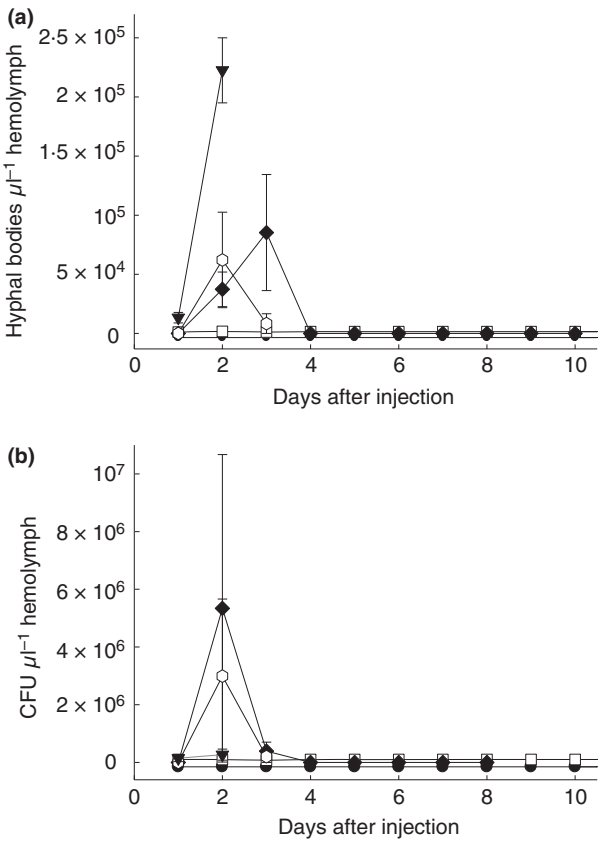

Figure 6 Mean number of hyphal bodies (HB) μl^{-1} haemolymph (± standard error of the mean, SE) (a) and mean number of Colony Forming Units (CFU) μl^{-1} haemolymph on culture medium (± SE) (b) of *Periplaneta americana* up to 10 days after injection of *Metarhizium anisopliae* HB ($2{\cdot}3 \times 10^3$ –●–, 6×10^3 –□–, 2×10^4 –♦–, 6×10^4 –◇– e 2×10^5 –▼– HB nymph^{-1}) and incubation at 25°C and 75 ± 5% humidity up to 10 days.

through an auto-grooming behaviour common in cockroaches, including *P. americana* (Böröczky *et al.* 2013; Zhukovskaya *et al.* 2013). The mutual grooming between different individuals reported in social insects that had been treated previously with conidia of entomopathogenic fungi or in the control (Yanagawa *et al.* 2009, 2010) was not detected in the present study. Conidia alone but also the emulsion without conidia stimulated the nymphs to intensify the cleaning activities of their body′s surface. It seems that *P. americana* nymphs are able to detect and to remove conidia of *M. anisopliae*. It might be assumed that nymphs ingested conidia present on the antennae and forelegs when passing them through their mouthparts, and it is possible that components in the saliva or gut secretion exert a fungistatic or fungitoxic action in these conidia in the gut. The mechanical and supposed physiological inactivation of

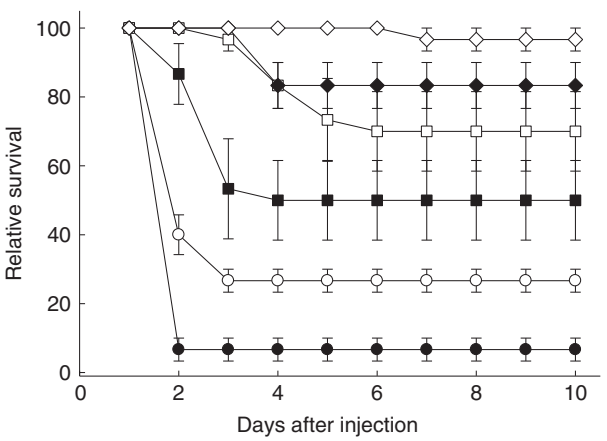

Figure 7 Survival of nymphs of *Periplaneta americana* up to 10 days after injection of *Metarhizium anisopliae* IP 46 hyphal bodies (control –◇–, 2×10^3 –♦–, 6×10^3 –□–, 2×10^4 –■–, 6×10^4 –○–, 2×10^5 –●– HB nymph^{-1}).

initially infective conidia on the cuticle and probably in the gut seems to be one of the reasons for the high resistance of *P. americana* against fungal infection.

Both the low number of nymphs with HB in the haemolymph as well as the low mortality after topical application of conidia emphasized the elevated resistance of late instar *P. americana* nymphs to infection by *M. anisopliae* and indicated that, in addition to the cuticular barrier, potent immune defence mechanisms in the cockroaches serve to protect against fungal infection. Up to the present time is not clear why only a few individuals were found with high numbers of HB or CFU. The results suggest that a successful infection through the cuticle takes about 3 days after topical application for *P. americana*, and that there is a critical period of an additional 5 days when HB are present and proliferating in the haemolymph but may be actively attacked by the host's immune system. Nymphs that survived for 8 days after topical application had probably overcome any earlier exposures to fungal conidia and HB, but there are no data to support this assumption.

The susceptibility of nymphs to infection by HB injections that bypass the cuticular barrier was dosage dependent. At lower doses, fungal propagules were probably mostly eliminated in the haemolymph by cellular immunity and enzymatic defence mechanisms, as has been reported in other insects (Zibaee *et al.* 2010; Chouvenc *et al.* 2011; Mirhaghparast *et al.* 2013); these cockroaches were not harmed by the infection. At higher doses, however, all nymphs succumbed quickly, and high numbers of HB were detected in the haemolymph before death.

Results about the mortality of nymphs treated topically with *M. anisopliae* conidia in this study emphasized the distinct resistance of older *P. americana* nymphs to infection by this fungus in comparison to younger nymphs and to adults, all of which were distinctly more susceptible to infection (Hubner-Campos *et al.* 2013). There is still no clear evidence that moulting by treated nymphs prevented any initial infections by *M. anisopliae* as was demonstrated for other hemi- and holometabolous insects exposed to *Beauveria bassiana* (Fargues and Vey 1974; Luz *et al.* 2003). Moreover, the reduced number of individuals killed by fungal topical applications developing on their surface in humid chambers suggests a certain difficulty of *M. anisopliae* to grow out of dead *P. americana* nymphs for the proliferation and dispersion of its conidia.

Injection of fungal blastospores circumvents the need for germination of conidia and penetration of the cuticle, and introduces a synchrony and magnitude of acute infection that is almost certainly absent when conidia are applied topically (Mullen and Goldsworthy 2006). In the present study, the blastopores there were only detected in the haemolymph and *in vitro* culture between the first and third day after injection. Differences in survival and infection of dose- response of nymphs treated with *M. anisopliae* blastospores emphasized the resistance of *P. americana* nymphs to infection by this fungus.

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Conflict of Interest

There are no conflicts of interest to declare.

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