2. Materials and methods

2.1 Material

2.1.1 Chemicals tested

Fenoxycarb is a juvenile hormone analogue with the empirical formula $C_{17}H_{19}O_4N$ and a molecular weight of 301.3 (Appendix 1).

Flufenoxuron is a chitin synthesis inhibitor with the empirical formula $C_{12}\ H_{11}\ Cl\ F_6\ N_2\ O_3$ and a molecular weight of 448.5 (Appendix 2).

Methanol was used as a solvent for both compounds as recommended by Janssen Pharmaceutica.

2.1.2 Origin of termites

Foraging termite workers used in this study were taken from laboratory colonies of *R. santonensis* collected at Ile d'Oléron, France, and then transferred and reared in BAM (Federal Institute for Materials Research and Testing), Berlin. The colonies were kept in moist vermiculite in a metal container (measuring 200 x 100 x 90 cm) at constant temperature and humidity (25 ± 1 °C; 75 ± 5%). From those colonies test termite workers were collected using rolled corrugated cardboard as baits. *Neotermes castaneus* were collected from Cuba in 1971. Reproductive pairs were reared in plastic containers measuring (15 x 11 x 11 cm) in moist vermiculite at constant temperature and humidity (25 ± 1 °C; 75 ± 5%).

Macrotermes bellicosus alates were collected from Ivory Coast (1992) during their swarming season. The reproductives pairs were reared in plastic containers measuring (30 x 25 x 35 cm) in moist soil at constant temperature and humidity (25 ± 1 °C; 75 ± 5%).

For the establishment of laboratory cultures, alates of *Microtermes* sp nr. *albopartitus* were collected during the rainy season (May-September) in Khartoum and Wad Medani (Sudan) in 1999 and 2000.

2.2. Laboratory experiments

2.2.1 Treatments of *R. santonensis* with fenoxycarb

To illustrate the effects of fenoxycarb on *R. santonensis*, four application methods were adopted. These were oral, contact, topical treatments and transferability by trophallaxis behaviour. The number of dead workers and those workers, which differentiated into presoldiers, was determined each day for each treatment. The dead termites were removed to avoid bacterial and fungal contamination. Treated filter papers were weighed after the treatment and at the end of the experiment when the filter papers were air-dried and their final weight loss was calculated. The consumed weight of the different treated filter papers was compared with that of the control filter papers in the same petri dish. The experiments were terminated when one or more of the following occurred:

i) Over 70% of the workers had moulted into presoldiers.
ii) The majority of the workers had become sluggish.
iii) The produced presoldiers had started to die.

Data were statistically analysed using non-parametric Kruskal-Wallis ANOVA due to non-normally distributed data. For single comparisons the Mann-Whitney U-test was used. Data of weight consumed were subjected to the Mann-Whitney-U test. Unistat statistical programme was used for the statistical analysis

2. 2.1.1 Oral treatment

Filter paper pads (Gelman Sciences, Germany) measuring 4.5 cm in diameter and 1 mm thick were divided into two equal halves. Each half was uniformly impregnated with 0.5 ml of the required concentration of fenoxycarb or methanol (as control). In order to avoid any adverse effect of alcohol on the termites, the treated filter papers were air dried for 24 hours for the methanol to evaporate. Groups of 30 workers were placed in a petri dish measuring 5 cm in diameter. Each petri dish contained 2 halves of a filter paper (Fig. 3), one half was always untreated and the other half was either treated with

methanol only (control) or with the active ingredient fenoxycarb at concentrations of 0.32 ppm, 1.6 ppm, 3.2 ppm, 16 ppm, or 32 ppm. There were 10 replicates for each treatment, i.e. a total of 1800 *R. santonensis* workers were used. Test termites were kept moist in constant darkness at 25 ±1 °C, and 75 ± 5% relative humidity. Experimental groups (Fig. 4) were checked every day for attractant or repellent effects of the treatment, filter paper consumption and morphological changes in the workers.

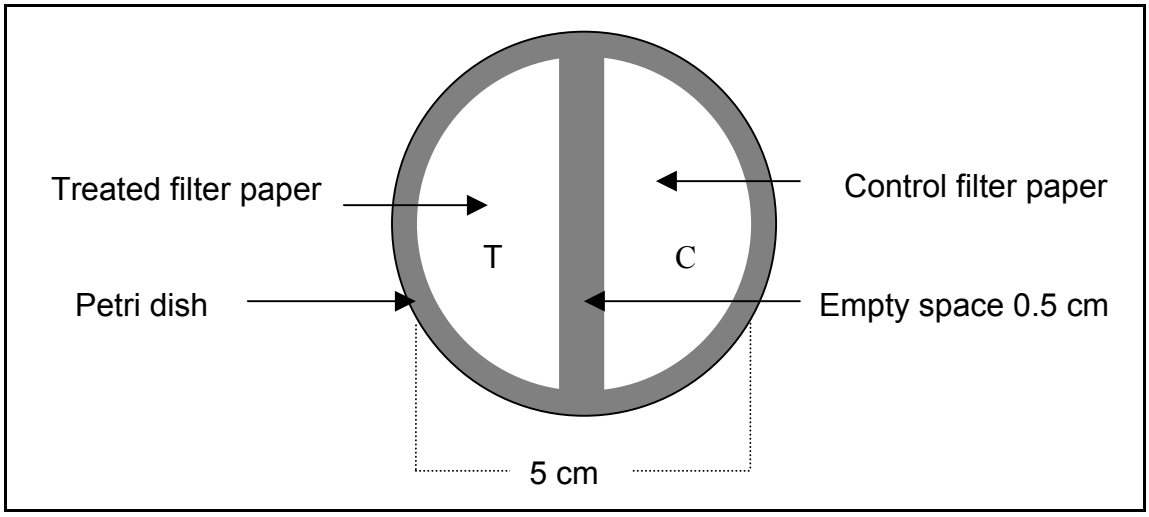

Fig. 3: Layout of experimental unit (oral treatment)

Fig. 4: Different castes of *R. santonensis;* from left to right: Queen, nymph, soldier, worker, larva. Experiments were performed with workers.

2. 2.1. 2 Contact treatment

Fenoxycarb solution in methanol (0.5 ml) of different concentrations was applied to half of the bottom of a petri dish (5 cm diameter) and the methanol was allowed to evaporate for 24 hrs. A filter pad moistened with 0.5 ml water, was placed on the other half of the petri dish to provide food to the termites (Fig. 5). Then, groups of 30 workers were introduced to the petri dish. The following concentrations were tested: 0.0 (control), 3.2 ppm or 32 ppm fenoxycarb. Each treatment was repeated 10 times. Experimental units were 30 workers x 3 concentrations x 10 replicates.

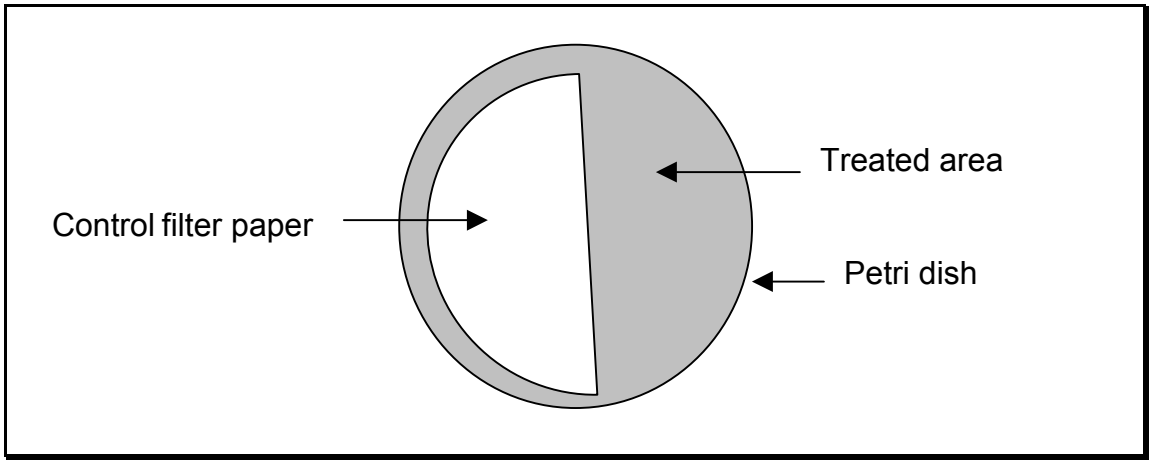

Fig. 5: Layout of experimental unit (contact treatment)

2.1.1.3 Topical treatment

Groups of 30 workers were treated topically on the tergum with 0.5 µl solutions of 32 ppm or 320 ppm fenoxycarb. Methanol alone was applied to the control termites. The solvent was released onto the dorsum of the workers abdomen with a micro-applicator (Instrumentation Specialities Companies, USA) fitted with a syringe and a fine glass needle. Treated workers were held up for thirty seconds for the methanol to evaporate, then placed in petri dishes measuring 5 cm in diameter. Untreated moistened filter paper was added to each petri dish as source of food. Each treatment was repeated 10 times, i.e. a total of 900 termites have been used.

2.2.1.4 Transferability of fenoxycarb by trophallaxis

In this experiment the transferability of fenoxycarb between nest mates of the colony was tested. Termites were either fed for 3 days on filter paper treated with fenoxycarb, or topically treated and immediately placed in 5 cm petri dishes. Untreated workers were added to the same petri dish. They were stained red by feeding them with filter papers treated with neutral red (2 % w/w) for three days.

Two concentrations, 32 ppm and 320 ppm of fenoxycarb, were used in different treatments as follows:

For oral treatment:

i) Ten workers treated with 32 ppm fenoxycarb + 30 workers dyed with neutral red.
ii) Twenty workers treated with 32 ppm fenoxycarb + 20 workers dyed with neutral red.
iii) Thirty workers treated with 32 ppm fenoxycarb + 10 workers dyed with neutral red.
iv) Twenty workers treated with 320 ppm fenoxycarb + 20 workers dyed with neutral red.

For topical treatment:

i) Ten workers treated with 320 ppm fenoxycarb + 30 workers dyed with neutral red.
ii) Twenty workers treated with 320 ppm fenoxycarb + 20 workers dyed with neutral red.
iii) Thirty workers treated with 320 ppm fenoxycarb + 10 workers dyed with neutral red.

There were 10 replicates of each treatment. The number of presoldiers produced determined the degree of transferability. The numbers of dead workers and those workers that differentiated into presoldiers were determined at 3 days' intervals for both treated and untreated workers for each combination.

2.2.2 Gas chromatographic (GC) analysis to determine uptake of fenoxycarb by the workers

In these tests the amount of fenoxycarb taken up by *N. castaneus* or *R. santonensis* workers were analysed on a Hewlett Packard 5890 gas chromatograph on a standard non polar column HP-35 crosslinked 35% PH ME Siloxane, equipped with NPD detector and operated in splitless mode. The inlet temperature was 250°C. The oven temperature was constantly at 230°C. Helium was used as carrier gas. Quantitative data were calculated on a chromatopac C-R-3-A integrator. Identification of the concentration was based on calibration using Tebuconazol as an internal standard.

For these tests, workers were collected in small tubes, to which 1 ml of toluene was added. The analyses were carried out at different time schedules as stated in the following sections. For each sample the substance was extracted using ultrasonic bath for 30 minutes. The preparation methods were mentioned in details in the specific sections.

2.2.2.1 Direct treatment

Termite workers were fed on filter papers treated with either 32 ppm or 320 ppm fenoxycarb following the method mentioned in section 2.2.1.1.

For contact treatment workers were put in petri dishes, half the bottom of which was treated with fenoxycarb using concentrations of 32 ppm or 320 ppm, while a filter pad was placed on the other half to provide food to the workers (section 2. 2.1. 2).

Treated termite workers were collected after 3, 6, 9 and 15 days for chemical analysis. For *N. castaneus* each test was performed using five workers, while for *R. santonensis* 20 workers were used. Five replicates were made for each treatment. The experimental design was made up of 2 concentrations x 2 application methods x 4 time intervals x 5 replicates.

2.2.2.2. Indirect treatment

In this experiment 600 workers of *R. santonensis* were divided into two groups. One group was topically treated with 3200 ppm fenoxycarb and the other one was treated with 1600 ppm fenoxycarb as described in section 2.1.1.3.

At the same time a batch of 600 workers was fed on neutral red for 3 days to attain the red coloration. Treated workers were divided into small groups of 20 for each concentration mixed with 20 untreated dyed workers in petri dishes measuring 5 cm in diameter. The amount of JHA taken up was measured using GC analysis. Treated workers and untreated ones were collected separately in glass tubes containing 1 ml toluene for the test after the following time intervals:

i) Immediately after the treatment.
ii) 1 day after the treatment.
iii) 2 days after the treatment.
iv) 3 days after the treatment.
v) 6 days after the treatment.

2.2.3. The effect of flufenoxuron on *R. santonensis*

2.2.3.1. Experimental procedures

The methodology of this experiment was similar to that of the fenoxycarb treatment given in section 2. 2.1.1. However, different concentrations of flufenoxuron were used. These were: 0 (control), 0.2, 1, 2, 10, 20 ppm flufenoxuron. Each concentration was tested 10 times.

The mortality rate was determined in a similar way as in section 2.2.1. Treated filter papers were weighed after the treatment and at the end of the experiment when the filter papers were air-dried and their final weight loss was calculated. The consumed weight of the different treated filter papers was compared with that of the control filter papers in the same petri dish.

Mortality data were statistically analysed using Kruskal-Wallis ANOVA. For single comparisons the Mann-Whitney U-test was used. Data of weight consumed were subjected to the Mann-Whitney U-test (non parametric test).

2.2.4. The synergistic effects of fenoxycarb and flufenoxuron on *R. santonensis*

In this bioassay, the effects of each of the following fenoxycarb and flufenoxuron combinations were tested. The general methods were the same as described in section 2.2.1. Each filter paper was impregnated with one of the following combinations:

i) 32 ppm fenoxycarb + 20 ppm flufenoxuron.
ii) 16 ppm fenoxycarb + 10 ppm flufenoxuron.
iii) Methanol only (control).

Based on the results obtained the following set of tests was conducted:

i) 32 ppm fenoxycarb + 0.02 ppm flufenoxuron.
ii) 32 ppm fenoxycarb + 0.2 ppm flufenoxuron.
iii) 32 ppm fenoxycarb +2 ppm flufenoxuron.

Furthermore, another set of tests was conducted with the following treatments:

i) 0.2 ppm flufenoxuron.
ii) 2 ppm flufenoxuron.
iii) 16 ppm fenoxycarb + 0.2 ppm flufenoxuron.
iv) 16 ppm fenoxycarb + 2 ppm flufenoxuron.

Each treatment was repeated 20 times. The workers, which differentiated into presoldiers were determined every day for each application as described in section 2.2.1.

2.3 The effect of fenoxycarb on laboratory colonies

2.3.1 The effect of fenoxycarb on *Microtermes* sp. nr*. albopartitus*

2.3.1.1 Colony establishment

Alates of *Microtermes* sp. nr*. albopartitus* were collected during the rainy season (May-September) in Khartoum and Wad Medani, Sudan, in 1999 and 2000. *M.* sp. nr*. albopartitus* swarms at approximately 6:50 p.m. following a previous rainy day. When alates emerged they had a short fly to a light source where they dropped to the ground and shed their wings. Males and females were attracted to each other by pheromones and form tandem pairs. Each couple then sets off, searching for a suitable nest site. Each couple was collected in a separate glass tube and taken to the laboratory. Petri dishes measuring 5 cm diameter were filled with sieved (500 μ mesh size) sterile soil. In each petri dish a hole was made in the centre to allow the alates to enter their artificial nest. Each collected couple was then put in a separate petri dish. The alates quickly entered through the prepared holes and the dishes were covered. Collected colonies were kept in an incubator at a temperature of 28 ± 1°C and moistened every day. Established colonies were taken to the BAM for further experiments.

2.3.1.2. Treatments

Filter papers measuring 15 mm in diameter were treated with 32 ppm fenoxycarb as described in section 2.1.1.1. After three months when the third instar nymphs emerged searching for food, the treatment was conducted. Foragers of test colonies were offered one filter paper (15 mm diameter) treated with 32 ppm together with one untreated filter paper. Control colonies received only untreated filter paper. To count the numbers of eggs and larvae, photographs were taken every three days from the window the termites had made at the bottom of the petri dish using a using a stereomicroscope equipped with a camera. Counting the absolute numbers of eggs and larvae from the photographs was not possible. Therefore, the following categories were used for quantification: 1-5, 5-10, 10-20, 20-30, 30-40. With these

categories, the median was calculated for treated and untreated colonies and compared using the Mann-Whitney U-test.

2.3.2. The effect of fenoxycarb on *Macrotermes bellicosus*

Grass, which is the preferred food of *Macrotermes bellicosus* was impregnated with 320 ppm fenoxycarb and fluorescent red dye. The treated grass was then offered to the colony. After six months the nest was opened and the observation was made regarding the presence of the eggs, larvae and the immature individuals. Photos were made using a digital camera for the follow up of the colony progress.

2.4. Acceptance of fenoxycarb and flufenoxuron in the field

2.4.1 Meteorology of the studied area in Sudan

The study was carried out in the year 2000. Three lawns at the University of Khartoum were selected for conducting these experiments (Fig 6).

According to The Sudan Meteorological Department the average annual rainfall is 161 mm and the mean temperature is 38° C. Temperature in Sudan increase in March to reach their first peak in May (42° C). After the rainy season, they rise again to reach a second peak in October (42° C), and then they decrease down to their minimum in January (6° C). The rainy season extends from May to October with the maximum amount of rain falling during July and August. The region is relatively dry and the mean relative humidity is 31%.

The lawns were covered with grasses and were heavily infested by termites. Irrigation was done by flooding. The prevailing termite species was *Microtermes* spp (sub-family Macrotermitinae), which are fungus-growing species.

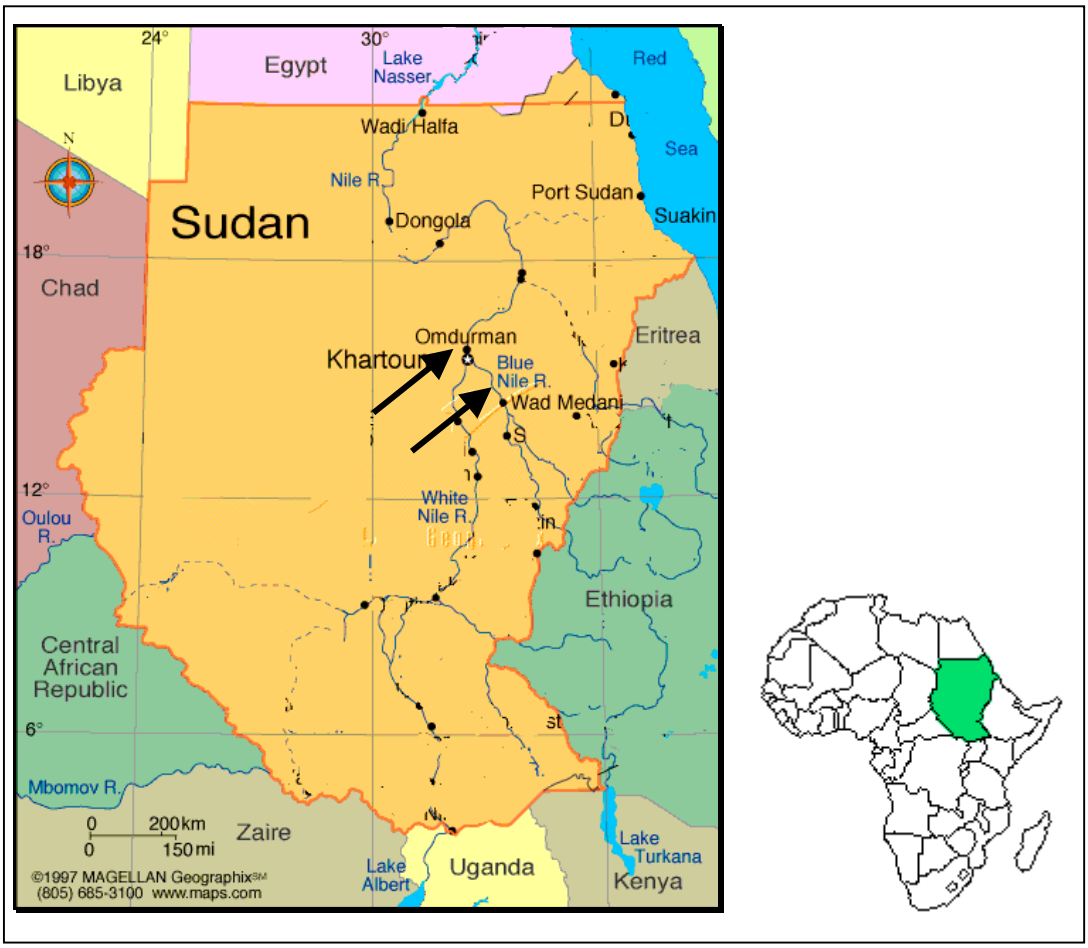

Fig. 6: Map of Sudan showing study area and sites (arrows) where *Microtermes sp. nr. albopartitus* was collected.

2.4.2 Treatment of the wooden baits

Soft wooden stakes (*Pinus sylvestris*) measuring 100 x 25 x 15 mm were vacuum impregnated with fenoxycarb, flufenoxuron, or methanol only, following the methods of the European standard EN 117 at concentrations of 32 ppm and 320 ppm for fenoxycarb and 20 ppm and 200 ppm for flufenoxuron. Wooden stakes were oven dried and weighed (± 0.1 g) before placement in the lawn.

2.4.3 Experimental layout

Before studying the effect of fenoxycarb and flufenoxuron on termites in the test fields, a three-week preliminary survey was conducted using untreated baits. Termites were collected at the baits for species identification.

In each lawn five concentrations were tested: 32 ppm and 320 ppm for fenoxycarb, 20 ppm and 200 ppm for flufenoxuron, in addition to the controls (methanol treated stakes). Five bait replicates for each compound and control were established in each plot. Thus, altogether 25 wooden stakes were distributed randomly in each lawn using Fisher's Latin square design (1968). The stakes were half buried in the soil in a 1 m x 1 m grid, or at intervals of 1 m apart around trees (Fig 7).

Fig 7: Experimental design with impregnated wooden baits (arrows) in the field.

2.4.4 Experimental observations

The baits were examined every three weeks. At each sampling occasion the following procedure was followed: Stakes were collected, taken to the laboratory where they were washed under running water to clean off the soil and debris. They were then oven dried, weighed and the consumed weight of each stake was recorded. Baits were replaced by another set of stakes treated with chemicals of the same concentrations.