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Effects of an Ecdysteroid Analog (RH-0345) on the Ovarian and Testicular Components of *Eupolybothrus nudicornis* (Myriapoda: Chilopoda)

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Abstract

We studied the effects of one ecdysteroid analog (RH-0345) on the ovarian and testicular components of the centipede *Eupolybothrus nudicornis* used as a model of predators of soil fauna. The injection of 10 µg of RH-0345 significantly reduced protein, lipid and carbohydrate concentrations in both the ovarian tissue of females and testicular tissue of males collected in spring or autumn. In consequence, this study shows that ecdysteroid analog pesticides can affect the reproduction of other arthropods than insects by modifying the level of metabolites in male and female gonads.

Keywords: Myriapoda, *Eupolybothrus nudicornis*, Ecdysteroid Analogs, Reproduction, Gonad Components, Carbohydrates, Proteins, Lipids

1. Introduction

Today, the use of pesticides in order to control pests causes much concern in the general population. Since it is virtually impossible to control all pest species without resorting to some use of pesticides, these are required to be specific and environmentally safe. In this context, natural and synthetic compounds capable of interfering with the processes of growth, development, reproduction and metamorphosis of the target pests have been developed in the search for safer insecticide technologies. These chemicals have been called insect growth regulators (IGRs) (Hoffmann and Lorenz, 1998). They work at extremely low levels, they are very specific and are not toxic to mammals; several compounds are already used. Among these compounds, novel insecticides that mimic the action of the molting hormone, the steroidal 20-hydroxyecdysone, are currently developed by the industry.

Bisacylhydrazines are non-steroidal ecdysteroid agonists of 20-hydroxyecdysone and exhibit their insecticidal activity via interaction with ecdysteroid receptor proteins (Dhadialla *et al.,* 1998). Although the effects of these compounds are well known in insects (Dhadialla *et al*., 1998), we know almost nothing about the effect of these compounds on other terrestrial arthropods. In previous studies, Daas *et al*. (2005 and 2007) used the centipede *Eupolybothrus nudicornis* (Gervais, 1837) (= *Eupolybothrus elongatus*, = *Bothropolys elongatus*) as a biological model to test the influence of ecdysteroid analogs on one of the predators of the soil fauna, this species could be directly affected by exposure to these compounds or affected following ingestion of preys containing these molecules. These authors showed that the injection of sublethal doses of two ecdysteroid analogs (RH-0345 or RH-5992), induced a significant decrease in hemolymph protein concentrations in males and females. A significant decrease in hemolymph lipid concentrations in males and females was also observed, except for females collected in spring and intoxicated with RH-0345 (Daas *et al*., 2005). Moreover, the injection of sublethal doses of RH-0345 and RH-2485 induced a significant reduction of total body and ovarian weights of female individuals (Daas *et al.,* 2007). Nevertheless, the decrease in ovarian weight was proportional to the decrease in total body weight because the gonadal somatic index remained constant and was the same among controls and intoxicated animals. These two compounds also reduced significantly the total protein concentrations in both the hemolymph fluid and ovarian tissue of females.

In order to better characterize the possible effect of ecdysteroid analogs on the reproduction of the centipede *E. nudicornis*, we tested the influence of RH-0345 on the ovarian and testicular components of individuals collected in spring or autumn.

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2. Materials and Methods

2.1. Animals and Housing

Experiments were conducted on mature females and males *Eupolybothrus nudicornis* collected during the spring or the autumn of 2008 from the area of Nechmaya (northeast Algeria) (Figure 1). This area is considered to be free of pollution. Animals were maintained individually in plastic boxes of 500 ml containing humid earth and covered with a moistened filter paper. They were fed three times a week with insects (cockroach larvae, flies and mosquitoes) and spiders.

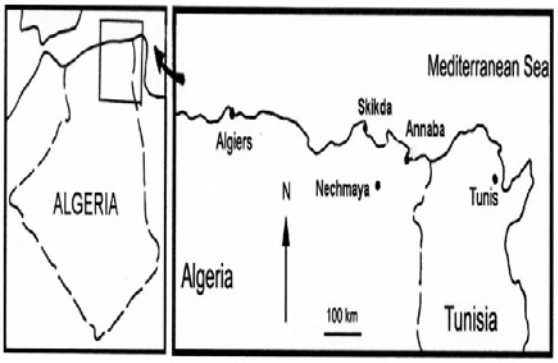

Figure 1. Map of Algeria showing the location of the site where individuals were collected

2.2. Chemicals And Toxicity Tests

RH-0345 (halofenozide) was developed by Rohm & Haas Company (Pennsylvania, USA). It was a gift by Prof. G. Smagghe (Laboratory of Agrozoology, University of Gent, Belgium). RH-0345 was dissolved in acetone to prepare a concentration of 3.33 µg/µl for experimental use. 3 µl were injected by means of a microsyringe between the third and fourth dorsal segment. Toxicity tests were performed during a 15-day period. Dose levels injected were based on previous finder range test (Daas *et al.*, 2005; 2007) and were chosen in order not to be lethal during the experiment. The amount of carbohydrates, lipids and proteins in ovaries and testicules were measured 5, 10, and 15 days after injection.

Control animals were maintained in the same conditions and injected with 3 µl of acetone.

2.3. Analytical Methods

Proteins, carbohydrates and lipids were extracted from the same gonad sample following the procedure of Shibko *et al.*, (1967). In brief, after dissection, each sample of gonad was individually homogenized in 1 ml of trichloroacetic acid (20%) and then centrifuged (5,000 g for 10 min at 4°C). The supernatant was used for the determination of carbohydrates as described by Duchateau and Florkin (1959) using anthrone as reagent and glucose as standard, while the pellet added with a mixture of ether and chloroform (1V/1V) was subjected to a second centrifugation (5.000 g for 10 min at 4°C). The resulted supernatant was used to quantify the lipids based on the vanillin method of Goldsworthy *et al.* (1972), Finally, protein concentration was determined in resulting pellet using the Bradford (1976) assay with bleu brilliant of coomassie (G 250, Merck) as reagent and bovine serum albumin (Sigma) as standard.

Data were expressed as µg of proteins, lipids or carbohydrates per mg of ovaries or testicules.

2.4. Data Analysis

The results were expressed as means ± standard deviation (S.D.). One-way ANOVA was used to compare the amounts of metabolites in ovaries and testicules from acetone injected animals (controls) and animals exposed to RH-0345 as a function of time. This analysis was followed by application of the Student-Newmans-Keuls multiple comparison method. The level of significance was set at $p < 0.05$.

3. Results

3.1. Ovarian Protein Concentrations

We obtained the same results with females collected in spring or autumn (Figures 2A and B). The ovarian protein concentrations of control females collected in spring or autumn remained stable the first 5 days and then slightly increased from 10 to 15 days but differences were no significant ($P>0.05$). The ovarian protein concentrations of females collected in spring were significantly ($P<0.05$) reduced compared to those of controls from 5 to 15 days after exposure (Figure 2A) whereas those of females collected in autumn were significantly ($P<0.05$) reduced from 10 to 15 days (Figure 2B). The ovarian protein concentrations were the same for females collected in spring or autumn.

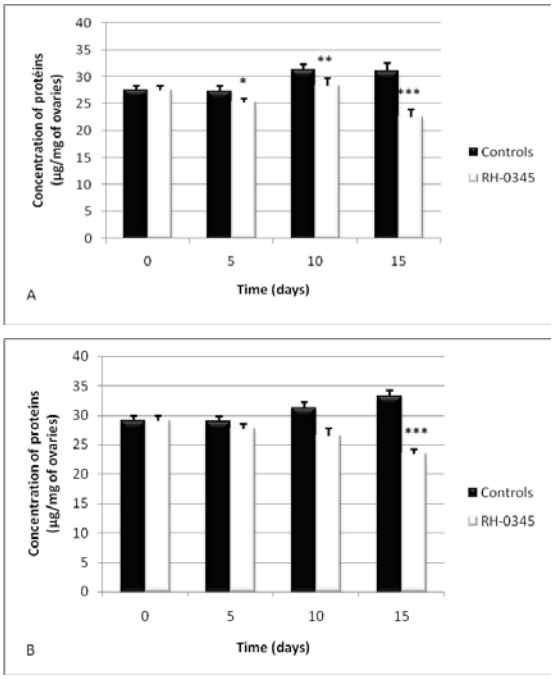

Figure 2. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* ovarian protein concentrations. A, females collected in spring; B, females collected in autumn. Means ± SD of 5 replicates. *$p < 0.05$; **$p<0.01$; ***$p<0.001$.

3.2. Testicular Protein Concentrations

The same results were obtained with males collected in spring or autumn (Figures 3A and B). The testicular protein concentrations of control males collected in spring or autumn decreased significantly (P<0.05) 5 days after beginning of the toxicity tests. Then we noticed an increase of the testicular protein concentrations 15 days and from 10 to 15 days after the start of the experiment for males collected in autumn and spring respectively. The testicular protein concentrations of males collected in spring or autumn were significantly reduced compared to those of controls from 5 to 15 days after exposure. No significant differences (P>0.05) were observed between testicular protein concentrations for males collected in spring or autumn.

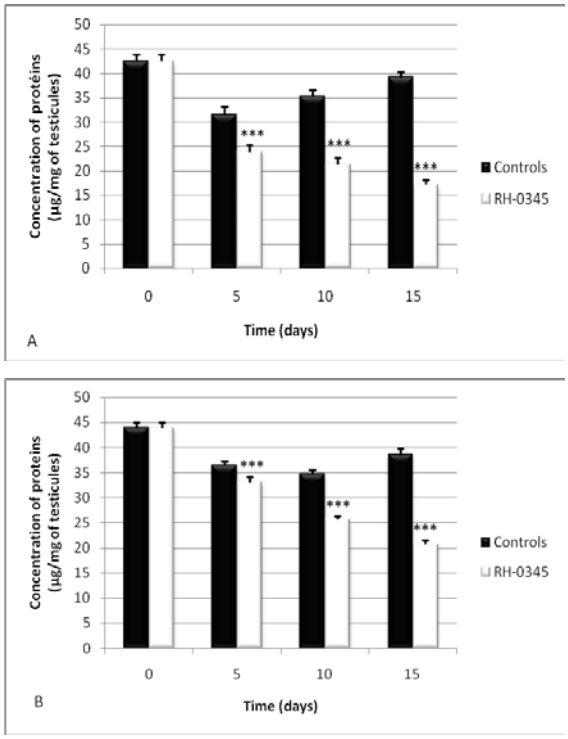

Figure 3. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* testicular protein concentrations. A, males collected in spring; B, males collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.3. Ovarian Lipid Concentrations

We obtained the same results with females collected in spring or autumn (Figures 4A and B). The ovarian lipid concentrations of control females slightly increased during the toxicity tests but differences were no significant (P>0.05). The ovarian lipid concentrations of females collected in spring or autumn were significantly (P<0.05) reduced compared to those of controls from 5 to 15 days after exposure. The ovarian lipid concentrations were the same for females collected in spring or autumn.

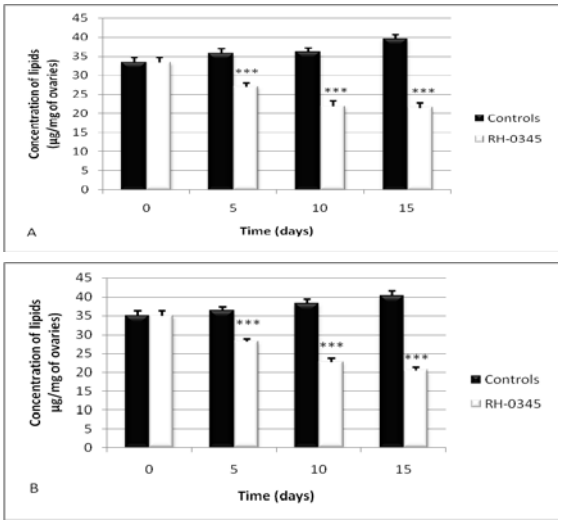

Figure 4. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* ovarian lipid concentrations. A, females collected in spring; B, females collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.4. Testicular Lipid Concentrations

The same results were obtained with males collected in spring or autumn (Figures 5A and B). The testicular lipid concentrations of control males decrease significantly (P<0.05) 5 and 10 days after the beginning of the experiment and then increased or remained stable for males collected in spring or autumn respectively. The testicular lipid concentrations of males collected in autumn (Figure 5B) were significantly reduced (P<0.05) compared to those of controls from 5 to 15 days after exposure whereas those of males collected in spring (Figure 5A) were significantly reduced from 10 to 15 days. No significant differences (P>0.05) were observed between testicular lipid concentrations for males collected in spring or autumn.

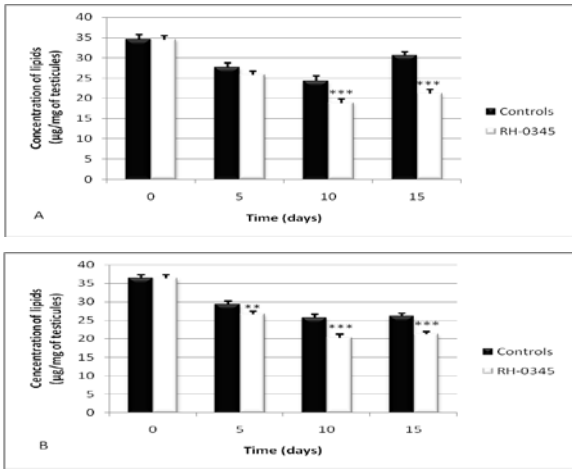

Figure 5. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* testicular lipid concentrations. A, males collected in spring; B, males collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.5. Ovarian Carbohydrate Concentrations

Globally we obtained the same results with females collected in spring or autumn (Figures 6A and B). The ovarian carbohydrate concentrations of control females

remained stable. The ovarian carbohydrate concentrations of females collected in spring or autumn were significantly reduced (P<0.05) compared to those of controls from 5 to 15 days after exposure. No significant differences (P>0.05) were observed between ovarian carbohydrate concentrations for females collected in spring or autumn.

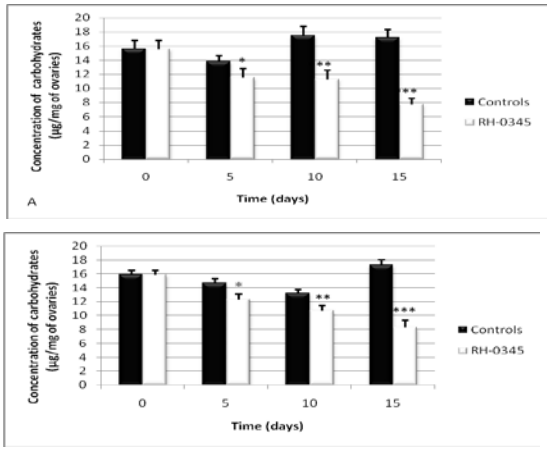

Figure 6. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* ovarian carbohydrate concentrations. A, females collected in spring; B, females collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

3.6. Testicular Carbohydrate Concentrations

Globally we obtained the same results with males collected in spring or autumn (Figures 7A and B). The testicular carbohydrayte concentrations of control males remained stable. The testicular carbohydrate concentrations of males collected in autumn (Figure 7B) were significantly reduced (P<0.05) compared to those of controls from 5 to 15 days after exposure whereas those of males collected in spring (Figure 7A) were significantly (P<0.05) reduced from 10 to 15 days. No significant differences (P>0.05) were observed between ovarian carbohydrate concentrations for females collected in spring or autumn.

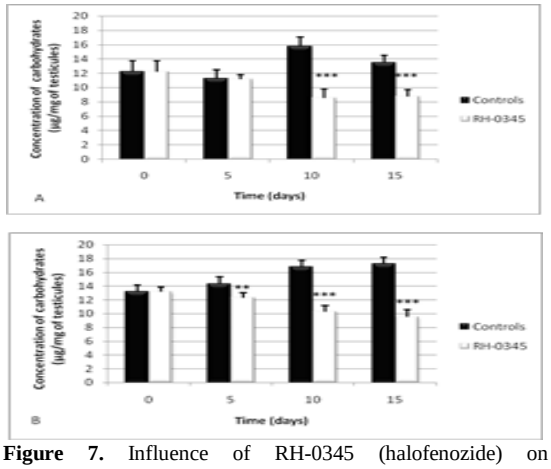

Figure 7. Influence of RH-0345 (halofenozide) on *Eupolybothrus nudicornis* testicular carbohydrate concentrations. A, males collected in spring; B, males collected in autumn. Means ± SD of 5 replicates. *p < 0.05; **p<0.01; ***p<0.001.

4. Discussion

The developmental physiology of myriapods as that of insects depends on different hormones and neurohormones but the molting and the juvenile hormones are the principal ones which control different processes such as growth, molt, and metamorphosis (Rees, 1995). Ecdysteroids which constitute the molting hormone have been considered as the main key in the control of the molt in immature stages of insects (Gäde *et al.*, 1997). Today, it is well accepted that ecdysteroids also play an important role in the processes which regulate the reproduction of insects such as meiotic reinitiation in oocytes, vitellogenesis, ovogenesis and growth of the spermatocytes (Wigglesworth, 1984; Hagedorn, 1985; Jacob, 1992; Lanot *et al.*, 1987; Yamashita and Susuki, 1991).

Hence, Robbins *et al.* (1968, 1970) reported that high concentrations of natural ecdysteroids had chemosterilizing properties in several insects. The goal of our study was to test the effects of one ecdysteroid analog (RH-0345) on the ovarian and testicular components of other arthropods than insects on which we have only limited evidence.

Bisacylhydrazines are non-steroidal agonists of 20-hydroxyecdysone (20E) and exhibit their insecticidal activity via interaction with the ecdysteroid receptor proteins. In cases where these compounds have produced lethal effects, the symptoms have been similar to those expected from a state of ecdysteroid excess, called hyperecdysonisms (Williams, 1967).

Proteins play a fundamental role in the functioning of the organism (Mahler and Cordes, 1969). They can assure the biochemical catalysis, the hormonal regulation and can be integrated in the cell as structural components just as lipids and sugars (Jacob and Monod, 1961). We showed that the ovarian and testicular protein concentrations of individuals (males or females) are significantly reduced after injection of a sub-lethal dose of RH-0345. These results are in accordance with previous studies of Taibi *et al.* (2003) who showed that ovarian protein concentrations of female adult beetles of mealworm *Tenebrio molitor* significantly decreased 2 and 4 days after topical treatment with 10 µg/insect of RH-0345. On the other hand, another ecdysteroid agonist, RH-5849 stimulates *in vitro* the protein synthesis by the fat 2body of the rice moth *Corcycra cephalonica* (Ashok and Dutta-Gupta, 1991). Maiza *et al.* (2004) showed that treatment of the German cockroach *Blattella germanica* with RH-0345 applied topically (10 and 20 µg/insect) and a carbamate insecticid benfuracarb orally administrated (at 2%) reduced ovarian amount of proteins while topical application of the juvenile hormone analogue methoprene (1 and 10 µg/insect) increased it during the sexual maturation.

Previous studies using certain steroid ecdysone analogs have shown that these compounds affected the ovarian growth in *Spodoptera exempta*, *S. exigua*, *S. littoralis* and *Leptinotarsa decemlineata* (Smagghe and Degheele 1992; 1994) and inhibited the ovarian development of *Musca domestica* and *Tribolium confusum* (Robbins *et al.*, 1968; 1970). Moreover, it has been shown that treatment of *Spodoptera exigua* and *Leptinotarsa decemlineata* with RH-0345 and RH-5992 induced a decrease of *hemolymphatic* protein concentrations before the death of individuals (Smagghe *et al.*, 1996). Nevertheless, treatment of the mealworm *T. molitor* with an analog of the juvenile hormone (pyriproxyfen) induced an increase of *hemolymphatic* protein concentrations (Aribi *et al.*, 2001; 2006). Besides, another growth regulator, RH-5992 (tebufonozide) reduced oocyte growth in *Plodia interpunctella* (Salem *et al.*, 1997).

Lipids are essential as a source of energy in arthropods (Beenakers *et al.*, 1985). They are synthetized and stored in the fat body (Keeley, 1985; Van Hensden and Law, 1989) and then transported *via* the hemolymph to organs such as ovaries (Kilby, 1963; Wigglesworth, 1984; Chino *et al.*, 1981) where they are used for vitellogenesis (Downer, 1985; Keeley, 1985). We showed that the ovarian and testicular lipid concentrations of individuals (males or females) were significantly reduced after injection of a sub-lethal dose of RH-0345.

During this study, we noticed that ovarian lipid concentrations of control females collected in spring or autumn increased during all the exposure time. On the contrary, we observed fluctuations of testicular lipid concentrations of control males collected in spring or autumn. These contrasting results could be explain by the change of the environmental conditions and the external factors whom probably induced physiological disturbances of reproduction and development as was demonstrated by Scheffel (1987) and Descamps (1988; 1992) during the study of the effect of weather conditions on the life cycle of another Chilopoda *Lithobius forficatus.*

The decrease of the lipid concentration in the ovaries of females after treatment with RH-0345 could be due to a slowing down of the passage of these metabolites towards ovaries via the hemolymph. Topical application of chitin inhibitors such as diflubenzuron at 0.5 µg/insect also disturbed growth and development of oocytes of the coding moth *Cydia pomonella* (Soltani and Soltani-Mazouni, 1992) and reduced lipid concentrations in the fat body of the mealworm *Tenebrio molitor* (Khebbeb *et al.*, 1997). Our results are in accordance with previous studies of Padjama and Rao (1994) who showed a decrease of lipid concentrations in viscera, mantle and foot of the freshwater snail *Bellamya dissimilis* after treatment with sublethal concentrations of an organochloric (Endosulfan) and three organophosphate pesticids (Methyl parathion, Quinalfos and Nuvan). Daas-Maamcha (2005) also showed that treatment of *E. nudicornis* females collected in spring or autumn with different ecdysteroid analogs (RH-2485, RH-5992 and RH-0345) induced a decrease of the protein and lipid concentrations in the hemolymph. A similar effect has been observed by Daas *et al.* (2003) after injection of 20-hydroxyecdyson to the females of the same species.

Carbohydrates which represent an indispensable source of energy for living organisms are used in a immediate way as glucose or in the form of reserve as glycogen (Wigglesworth, 1984). Tissular carbohydrate rates are strictly connected to the physiological events such as molt and reproduction (Wiens and Gilbert, 1968). We showed that the ovarian and testicular carbohydrate concentrations of individuals (males or females) were significantly reduced after injection of a sub-lethal dose of RH-0345. Soltani (1990) noticed a significant decrease of carbohydrates (trehalose) concentration in the hemolymph of pupal stages of mealworms *T. molitor* 3, 4, 5, 6 and 7 days after injection of 10 µg of 20-hydroxyecdysone

It is well recognised that ecdysteroid analogs are toxic to insects. The synthetic nonsteroidal ecdysone agonist RH-0345 is an excellent insect control agent because it induces feeding inhibition and precocious incomplete molting, thus causing high larval mortality (Dhadialla *et al.*, 1998). RH-0345 has an overall insect control spectrum with accentuated soil-systemic efficacy against scarabid beetle larvae, cutworms, and webworms. Based on its reported narrow pest control spectrum and its structural and mechanistic similarity to the other bisacylhydrazines, it is expected to have low toxicity to non-target arthropods (Dhadialla *et al.*, 1998). Nevertheless, our study showed that this pesticide may affect the reproduction of other arthropods than insects such as centipedes.

The decrease of protein, lipid and carbohydrate concentrations in both the ovarian tissue of females and testicular tissue of males after treatment with RH-0345 may be explain by the interference between this compound and natural ecdysteroids thus disturbing the endocrine regulation of oogenesis and spermatogenesis as in *Tenebrio molitor* (Amrani, 2007; Boukachabia *et al.*, 2003; Taibi *et al.*, 2003).

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