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Hellenic Plant Protection Journal **4:** 31-34, 2011

SHORT COMMUNICATION

Report of the Geranium Bronze Butterfly, *Cacyreus marshalli* for mainland Greece

A.F. Martinou[1], D. Papachristos[2] and P.G. Milonas[1]

Summary In July and September 2010, two samples of infested geranium plants (*Pelargonium* spp.), which were originally collected from Kifissia, Attica-Greece, were received at the Laboratory of Biological Control at Benaki Phytopathological Institute, Greece. Larvae were taken from infested plants and kept under laboratory conditions at 25±1°C, 70±5% RH and under a photoperiod of 16L:8D h until adults emerged. Adults were identified as the Geranium Bronze Butterfly, *Cacyreus marshalli* Butler (Lepidoptera: Lycaenidae). This species is recorded for the first time for mainland Greece. *Cacyreus marshalli* is on the EPPO A2 List of pests recommended for the regulation as quarantine pests. Geranium Bronze Butterfly has the potential to establish in Greece and the rest of the Mediterranean basin as climatic conditions can allow this pest to overwinter outdoors and its host plants are commonly propagated.

Additional keywords: biodiversity, butterfly, geranium, invasive, *Pelargonium* spp.

The identification of the species was based on two infested geranium plant samples received at the Laboratory of Biological Control at Benaki Phytopathological Institute in July and September 2010. The plant samples (leaves and stems) were originally collected from Kifissia, Attica-Greece and were kept at 25±1°C and 70±5% RH until adult emergence. Based on the adult morphological characteristics, the species was identified as the Geranium Bronze Butterfly *Cacyreus marshalli* (Lepidoptera: Lycaenidae) (Figures 1, 2). Eggs of the Geranium Bronze are whitish to light-yellow or brown in colour (Figure 3); 0.5 mm in diameter x 0.3 mm in height (2). Eggs are laid near the flower buds or less frequently on the leaves. Caterpillars (Figure 4) are found within the flower buds or inside the stem, where they bore through. Entrance holes in buds and stems are easy to detect. Once attacked, the stems turn black-

Figure 1. Adult of *Cacyreus marshalli* (upperside).

Figure 2. Adult of *Cacyreus marshalli* (underside).

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Figure 3. Egg of *Cacyreus marshalli.*

Figure 4. Caterpillars of *Cacyreus marshalli.*

ish. The first- instar larvae have an average length of 1 mm which increases to 2 mm within 8 days. Second, third and fourth- instars grow to 3, 6 and 13 mm, typically in 8, 8 and 9 days, respectively. Their colour varies, with extremes of yellow and/or greenish shades with or without pink markings (2). Pupae are very hairy in shades of green, pale-yellow or brown, with brown mottling and an average length of 9 mm (2). Female adults have a wingspan of 18-27 mm while male adults have a wingspan of 15-23 mm. The two sexes are similar in appearance. Adults have a bronze-brown colouring at the upper surface with white spots on the fringe and highly-patterned undersides. They also bear substantial tails on their hindwings, along with a nearby eye spot, which diverts attacks from birds and other predators away from the critical body parts.

The species is indigenous to Southern Africa (2). According to the EPPO distribution maps of quarantine pests, *C. marshalli* is present (with no detailed records) in Botswana, Lesotho, Mozambique, South Africa, Swaziland and Zimbabwe. To date it has been observed in European countries such as Belgium and Italy with a few occurrences, France and mainland Spain, where it is recorded as present with a restricted distribution, and finally Germany, Portugal and the Balearic and Canary islands, where it is reported as present but with no detailed records. This is the first record for mainland Greece while there are previous records for the species from the Ionian island of Corfu (7, 10).

Pelargonium spp., commonly known as geraniums, are the main host plants of this pest but the butterfly also has the capacity to infest native *Geranium* spp. (8).

Cacyreus marshalli is on the EPPO A2 List of pests recommended for regulation as quarantine pests. Although on the EPPO A2 List, *C. marshalli* has not been regulated as a quarantine pest by European Regional Plant Protection Organizations and it has managed to spread rapidly in islands and mainland Spain and Italy (4, 9). The potential for natural spread of this pest is very low as its flights are short in duration with frequent rests (3). The most likely means of international dispersal is the movement of infested plant material. The example of the rapid establishment of *C. marshalli* on Mallorca (Balearic Islands) and its spread to the Spanish mainland shows that the pest has the potential to establish in the Mediterranean basin and could pose a threat for the European mainland. Geraniums are extensively grown as ornamental plants almost throughout Europe, but Spain, France, Italy and Greece, as well as North Africa, are at greater risk since their climatic conditions would allow the pest to overwinter outdoors. Furthermore, breeding and propagation of geraniums play an important role in these regions. Elsewhere in Europe, the pest could establish in glasshouses (1).

Cacyreus marshalli has never been re-

ported as a pest species in its area of origin, probably due to autochthonous parasitoids and predators that manage to keep its population under the damage threshold. The introduction of the Geranium Bronze into Europe is having a great impact on the nursery sector, with a consequent decrease in the demand of geraniums, which are ever more often replaced by customers with other ornamental plants. Furthermore, the Geranium Bronze could cause problems in the mountainous and hilly habitats where wild *Geranium* spp. commonly exist. Adaptation of *C. marshalli* in these habitats could threaten the native flora and biodiversity through competition with other species such as *Eumedonia eumedon* (Esper) (Lepidoptera: Lycaenidae) and *Aricia nicias* (Meigen) (Lepidoptera: Lycaneidae) (8). *Aricia nicias* has been characterized as rare for Spain (5) while *E. eumedon* has been characterized as vulnerable in Greece (11).

No parasitoids or predators were observed in Italy for *C. marshalli* (4) except of a single case where an egg of the pest was parasitized by *Trichogramma evanescens* Westwood. In South Africa, *Apanteles* spp. have been reported to kill third- instar larvae of the pest (2). Products based on *Bacillus thuringiensis* or diflubenzuron, flufenoxuron, hexaflumuron, lambda-cyhalothrin, α-cypermethrin and benfuracarb could be used as insecticides for the control of *C. marshalli* (9). For the long-term control of the Geranium Bronze with *B. thuringiensis*-based insecticides it would be advisable to combine Cry1Ab with Cry1Ba (6). It would be wise to start a monitoring programme for this pest in *Pelargonium* spp. nurseries as well as in urban resident areas of mainland Greece and the islands where these plants are widely used in gardens and in balconies. Additionally, surveys could be undertaken in the monitored areas in order to identify predators or parasitoids that could be used as potential biocontrol agents for this pest.

We would like to thank Mr Timothy Cowles for providing with photos and Mr Rob Parker for sharing with us his interest on butterflies, especially regarding the Geranium Bronze Butterfly.

Literature cited

1. Baufeld, P. 1993. Pest risk analysis of *Cacyreus marshalli* from a phytosanitary point of view. *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes,* 45: 257-262.
2. Clark, G.C. and Dickson, C.G.C. 1971. *Life histories of the South African lycaenid butterflies*, pp. 60-61. Purnell, Cape Town, South Africa.
3. Eitschberger, U. and Stamer, P. 1990. *Cacyreus marshalli*, a new species of butterfly for the fauna of Europe? *Atalanta,* 21: 101-108.
4. Favilli, L. and Manganelli, G. 2006. Life history of *Cacyreus marshalli*, a South African species recently introduced into Italy. *Bollettino della Societa Entomologica Italiana,* 138: 51–61.
5. Gärdenfors, U. 2005. Rödlistade arter i Sverige 2005. The 2005 Red List of Swedish Species. 496 pp. ArtDatabanken, SLU, Uppsala.
6. Herrero, S., Borja, M. and Ferre´, J. 2002. Extent of Variation of the *Bacillus thuringiensis* Toxin Reservoir: the Case of the Geranium Bronze, *Cacyreus marshalli* Butler (Lepidoptera: Lycaenidae). *Applied and Environmental Microbiology,* 68: 4090–4094.
7. Parker, R. 2010. *Cacyreus mashalli* Butler, 1898 (Lepidoptera, Lycaenidae) newly recorded for Corfu, with notes on other butterflies on the island in September 2008. *Entomologist's Gazette,* 61: 40-42.
8. Quacchia, A., Ferracini, C., Bonelli, S., Balletto, E. and Alma, A. 2008. Can the Geranium Bronze, *Cacyreus marshalli*, become a threat for European biodiversity? *Biodiversity and Conservation,* 17: 1429-1437.

Web sites

9. http://dc.eppo.org/
10. http://www.pamperis.gr/THE_BUTTERFLIES_OF_GREECE/Nees_anaphores_New_records.html
11. http://filotis.itia.ntua.gr/species/d/6642/5. Gärdenfors

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Αναφορά του *Cacyreus marshalli* στην Ηπειρωτική Ελλάδα

Α.Φ. Μαρτίνου, Δ. Παπαχρήστος και Π.Γ. Μυλωνάς

Περίληψη Τους μήνες Ιούλιο και Σεπτέμβριο του 2010, δύο δείγματα από προσβεβλημένα γεράνια (*Pelargonium* spp.) από την περιοχή της Κηφισιάς εξετάστηκαν στο εργαστήριο Βιολογικής Καταπολέμησης στο Μπενάκειο Φυτοπαθολογικό Ινστιτούτο. Οι προνύμφες από τα προσβεβλημένα φυτά διατηρήθηκαν σε συνθήκες εργαστηρίου στους 25±1°C, 70±5% σ.υ. και φωτοπερίοδο 16Φ:8Σ μέχρι την εμφάνιση των ενηλίκων. Τα ενήλικα αναγνωρίστηκαν ως το είδος *Cacyreus marshalli* Butler (Lepidoptera: Lycaenidae). Το είδος αυτό καταγράφεται για πρώτη φορά στην Ηπειρωτική Ελλάδα. Το *C. marshalli* βρίσκεται στην EPPO A2 λίστα οργανισμών καραντίνας. Το λεπιδόπτερο δύναται να εγκατασταθεί στην Ελλάδα και στην υπόλοιπη Μεσογειακή λεκάνη καθώς οι κλιματολογικές συνθήκες ευνοούν τη διαχείμαση του ενώ τα φυτά ξενιστές του είναι πολύ κοινά.

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Larvicidal evaluation of three *Mentha* species essential oils and their isolated major components against the West Nile virus mosquito

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Summary The larvicidal activity of essential oils derived from three different *Mentha* species (Lamiaceae) as well as their major aroma *p*-menthane type components were evaluated against *Culex pipiens* (Diptera: Culicidae). Therefore, pulegone, piperitenone, piperitone, carvone, menthone and menthol were isolated using column chromatography. The LC_{50} values revealed that *M. pulegium* and *M. piperita* oils were the most toxic (46.97 and 40.28 mgl^{-1} respectively) and pulegone was the most effective (27.23 mgl^{-1}) among the major ingredients. The activity of all essential oils is in agreement with the proportion/toxicity rate of their individual major components, apart from the case of *M. piperita* where the LC_{50} values of its major ingredients menthone and menthol stand higher than 100 mgl^{-1}. For the isolated molecules, studies on structure activity relationships revealed that the location of C-C double bond and the presence of the isopropylidene group might be key factors.

Additional keywords: Culex pipiens, essential oil, larvicidal activity, major components isolation, *Mentha* species

Introduction

Control of mosquitoes is crucial due to the fact that they are vectors of many viruses. *Culex pipiens* serves as both an enzootic and an epidemic (i.e. "bridge") vector of West Nile Virus (WNV) to humans, is a wide spread insect pest with medical importance. According to Bakonyi et al. (4) WNV emerged in several European countries within the last 50 years. Outbreaks of WNV encephalitis in humans and horses were reported. Recently, new WNV strains were isolated in Central Europe from mosquito vectors and from encephalitic cases of vertebrate host (18). During the last decade cases are also reported from Russia, Israel, Turkey and other Mediterranean countries (10). During summer of 2010, hundreds of people were seriously affected from WNV and more than 30 died in Northern Greece (18). Currently specific treatment and vaccines are not available for the protection of horses or humans and only mosquito control measures could reduce the risk of the development of serious diseases (6). Therefore the use of improved mosquito control measures is strongly emphasized. According to Floore (11) the success of mosquito control relies on product efficacy and tools that are environmentally friendly. Shaalan et al. (21) stated that ".. the failure to discover a significant new class of insecticides has led many researchers back to biodiscovery studies…". Plant derived pesticides are biodegradable and may be the future arsenal against mosquitoes but there are many topics that need more investigation.

Previous investigations have indicated that various *Mentha* spp. plant extracts displayed larvicidal effect on *Cx. pipiens*, *Cx. quinquefasciatus*, *Aedes aegypti, Anopheles stephensi* and *An. tesselatus* (2, 3, 19, 20, 22, 23). Furthermore, pure substances (thymol, menthone, menthol and pulegone) and menthol derivatives have been tested against mosqui-

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to adults, while menthone, thymol and carvacrol against *Culex* species for larvicidal activity (20, 23). These substances are known active ingredients not only of *Mentha* species essential oils but also of many other plants, in which their concentrations vary (7).

Main objectives of this study were: a) to evaluate the efficacy of essential oils derived from three *Mentha* species, *M. pulegium* (three populations), *M. piperita* and *M. spicata* (spearmint) against *Cx. pipiens* larvae, b) to isolate and evaluate essential oil major components, c) to correlate larvicidal activity of isolated components with their precursor oils and d) to study the structure activity relationships for the isolated molecules.

Materials and Methods

Plant Materials

Three different species of the genus *Mentha* were used: *M. pulegium* (pennyroyal), *M. piperita* (mint) and *M. spicata* (spearmint). All plant materials were collected from various natural habitats in Greece by members of our team, between July and August 2007 in the flowering stage. Specifically, in the case of *M. pulegium* samples were collected from three populations according to their geographical origin, the first one from Orestiada (North-East of Greece, named as PUL1), the second from Karditsa (center of Greece, PUL2) and the third from Heraklion (Island of Crete, PUL3). Samples of *M. spicata* (SP) and *M. piperita* (PIP) were purchased from local farmers from the region of Karditsa. All five plant materials were air dried and stocked for further use.

Chemicals

R-(+)-pulegone,terpinen-4-ol,*β*-farnesene, *α*-pinene, *β*-pinene, mesityl oxide, methyl vinyl ketone, menthol and menthone were purchased from Aldrich (Steinheim, Germany). Eucalyptol, *β*-myrcene, *S*-(-)-limonene, *γ*-terpinene, thymol, carvacrol and *β*-caryophyllene were purchased from Sigma (St. Louis, USA). Carvone was bought from Fluka (Steinheim, Germany). 3-methyl cyclohexanone were bought from Jansen Chimica (Beerse, Belgium). Terpinolene, *iso*-menthone and piperitone were purchased from Extra Synthese (Genay, France). Diethyl ether (BHT free) was purchased from SDS (Cedex, France). Pentane was bought from Lab-Scan (Doublin, Ireland). Piperitenone was prepared according to literature synthetic procedure (5), due to commercial unavailability. Purity of the isolated product (97.3%) was estimated according to GC-MS analytical conditions given bellow. Structural characterization that was accomplished by mass spectral analysis and NMR experiments was in agreement to literature data (5). NMR spectra were recorded on Brucer Avance DRX-500 instrument. Potassium hydroxide, tetrahydrofuran (THF), silica gel 60G and TLC plates (silica gel 60, F254) were purchased from Merck (Darmstadt, Germany).

Isolation of the Essential Oils

Aerial parts from PUL1, PUL2, PUL3, SP and PIP were powdered in an electrical blender and 1 Kg of each sample submitted to hydrodistillation for 4 h in a Clevenger-type apparatus. The obtained essential oils, named as EOpul1, EOpul2, EOpul3, EOsp and EOpip respectively, were dried over anhydrous magnesium sulfate. After filtration their volume were calculated and expressed as ml of essential oil/100 g of dry material (Table 1) and finally stored in labeled sterile screw capped bottles at -22°C until use.

Gas chromatography – Mass spectrometry (GC-MS) analysis

The essential oils were analyzed using a Hewlett Packard II 5890 gas chromatography (GC) system, equipped with a FID detector and HP-5ms capillary column (30 m x 0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were set at 220°C and 290°C, respectively. GC oven temperature was programmed from 60°C to 240°C at a rate of 3°C /min and held isothermally for 10 min. Helium was the carrier gas at a flow rate of 1 ml/min. Diluted samples (1/100 in diethyl ether, mgl^{-1}) of 1.0 μl were injected manually and in the splitless mode. Quan-

titative data were obtained electronically from FID area percent data without the use of correction factors. Qualitative analysis of the essential oils was performed using the same conditions with GC, in a Hewlett Packard II 5890 gas chromatograph equipped with Hewlett Packard II 5972 mass selective detector in the electron impact mode (70 eV). Most ingredients of the essential oils were identified on the basis of comparison of GC relative retention times and mass spectra with those of pure standards. As for the rest components, tentative identification was based on the comparison of their mass spectra and elution order with those obtained from the NIST 98 and Wiley 275 library data of the GC-MS system and from Adams CD computer library (1).

Isolation of Essential Oils Major Ingredients

Ten (10) g of the extracted crude essential oils EOpul1, EOpul2, EOpul3, EOpip and EOsp were fractioned by column chromatography on silica gel and eluted with a gradient of solvents of increasing polarity (pentane + diethyl ether: 100 + 0, 97 + 3, 95 + 5, 93 + 7, 90 + 10 and 80 + 20). Column fractions were monitored by thin layer chromatography (TLC) with pentane + diethyl ether (9.5 + 0.5). Fractions with similar R_f values on the TLC plates to authentic co-eluted compounds were pooled. Spots were detected under UV lamp and afterwards by spraying with a mixture of 1% vanillin and 5% sulfuric acid solution (in ethanol) and heating at 120°C and/or PMA solution (phosphomolybdic acid 7.5% mgl^{-1} in ethanol) and charring on a hot plate. The chosen fractions merged to one and elution solvents removed by a rotary flash evaporator. The distillation was stopped when the volume of solvents was reduced to ~10 ml and completed by flushing through nitrogen.

Mosquito Rearing

The colony of the species *Cx. pipiens* biotype *molestus* has been maintained in the laboratory of Benaki Phytopathological Institute, Kifissia, Greece for more than 25 years. Adult mosquitoes are kept in wooden framed cages (33x33x33 cm) with a 32x32 mesh at 25±2°C, 80±2% relative humidity and photoperiod of 14:10 (L:D) h. Cotton wicks saturated with 10% sucrose solution are used as food source. Females lay eggs in round, plastic containers (10 cm diameter x 5 cm depth) filled with 150 ml of tap water. Egg rafts are removed daily and placed in cylindrical enamel pans (with diameter of 35 cm and 10 cm deep), in order to hatch. Larvae are reared under the same conditions of temperature and light and are fed daily with baby fish food (TetraMin, Baby Fish Food) at a concentration of 0.25 gl^{-1} of water until pupation. Pupae are then collected and introduced into the adult rearing cages.

Larvicidal Bioassays

Stock solutions were prepared in ethanol with a concentration of 1% mgl^{-1}. A series of aqueous solutions of the tested material, expressed as mgl^{-1}, were made and tested under laboratory conditions. Preliminary experiments (data not shown) were performed to evaluate toxicity and subsequently different concentrations were employed ranging from 10 to 200 mgl^{-1} for each tested material.

The larval mortality bioassays were carried out according to the test method of larval susceptibility as proposed by the World Health Organization (24). Twenty 3rd to 4th instar larvae of the species *Cx. pipiens* biotype *molestus* were collected from the colony. They were placed in glass beaker with 250 ml of aqueous suspension of tested material at various concentrations and an emulsifier was added in the final test solution (less than 0.05%). Four replicates were made per concentration and a control treatment with tap water and emulsifier was also included. Beakers with larvae were placed at 25±2°C, 80±2% relative humidity and photoperiod of 14:10 (L:D) h.

Data Analysis

Larvicidal effect was recorded 48 h after treatment. Data obtained from each dose–larvicidal bioassay (total mortality, mgl^{-1} concentration in water) were subjected to probit analysis in which probit–transformed

mortality was regressed against log_{10}–transformed dose; LC_{50}, LC_{90} values, and slopes were calculated (SPSS 11.0).

Results

Chemical Composition of the Essential Oils

Yields and chemical composition of the obtained essential oils are shown in Table 1. Literature data suggests that *M. pulegium* is a chemical polymorph species both in qualitative and/or quantitative composition. Recently, it has been demonstrated the link between the oil composition and origin of 38 populations of *M. pulegium* scattered along Greece (12). Thus, the three different samples resulted in different chemical profiles. In detailed, in EOpul1 pulegone (61.1%) was

Table 1. Percentage composition of essential oils isolated from *M. pulegium* (three populations: EOpul1, EOpul2 and EOpul3), *M. spicata* (EOsp) and *M. piperita* (EOpip). Compounds are listed in order of elution from an HP-5 MS column.

Components	Composition (%)				
	EOpul1[c]	EOpul2[c]	EOpul3	EOsp	EOpip[c]
3-methyl cyclohexanone[a]	0.4				
α-pinene[a]			0.2	0.4	0.7
β-pinene[a]			0.2	0.2	1.2
3-octanol[a]	1.4	0.4	0.7	0.2	
limonene[a]	1.2	2.7	1.8		
eucalyptol[a]				9.0	6.9
γ-terpinene[a]				0.4	0.7
menthone[a*]	4.3	1.8	1.6		**39.0**
isomenthone[a*]	13.0	0.3	**24.8**		9.9
menthol[a*]					**25.9**
terpinen-4-ol[a]				0.7	
cis-dihydro carvone[b]				1.0	
isopulegone[b]	2.9				
trans-carveol[b]				0.8	
pulegone[a*]	**61.1**				2.0
carvone[a*]				**71.8**	
piperitone[a*]	1.1	**92.6**	**69.3**		1.3
piperitenone[a*]	4.0	0.1	0.4		
piperitenone oxide[b]	1.8				
β-bourbonene[b]	0.1	0.8	0.2	1.5	0.4
β-elemene[b]				1.1	0.2
β-caryophyllene[a]				2.2	3.5
β-farnesene[a]				0.1	0.6
germacrene D[b]	0.1			1.3	2.9
bicyclogermacrene[b]				0.8	0.5
caryophyllene oxide[a]				0.2	
Total	**91.4**	**98.7**	**99.2**	**91.7**	**95.7**
oil yield (ml/100 dry wt)	2.2	2.1	2.1	1.8	3.2

[a] Comparison with pure standards.
[b] Tentative identification based on data obtained from NIST 98 and Wiley 275 library of the GC-MS system and from Adams CD computer library (16).
[c] For these essential oils the phytochemical analysis for major ingredients (>1%) has already been published (14).
* Components that were isolated and applied in bioassays.

the most abundant ingredient followed by isomenthone (13.0%), menthone (4.3%), piperitenone (4.0%), isopulegone (2.9%) and piperitenone oxide (1.8%). EOpul2 characterized by the dominant occurrence of piperitone (92.6%) and the minor presence of limonene (2.7%) and menthone (1.8%). Finally, EOpul3 consisted of piperitone (69.3%), isomenthone (24.8%), limonene (1.8%) and menthone (1.6%). Concerning the other two plants, the major ingredient of EOsp was carvone (71.8%) followed by eucalyptol (9.0%) and β-caryophyllene (2.2%) among others, while EOpip consisted of menthone (39.0%) and menthol (25.9%) with minor components isomenthone (9.9%), eucalyptol (6.9%) and β-caryophyllene (3.5%) among other less abundant ingredients.

Purity of the isolated major ingredients

The purity of the isolated components was determined by GC-MS analysis and is given as follows: pulegone (5.45 g, 99.2% purity) and piperitenone (0.38 g, 98.6% purity) from EOpul1, piperitone (8.63 g, 99.5% purity) from EOpul2, piperitone (6.21 g, 99.4% purity) and iso-menthone (1.86 g, 98.9% purity) from EOpul3, carvone (6.05 g, 99.6% purity) from EOpip and menthone (3.16 g, 99.2% purity) and menthol (2.17 g, 99.0% purity) from EOsp. Pulegone, piperitenone, piperitone, isomenthone, carvone, menthone and menthol have been isolated in high purities and their chemical structures are given in Figure 1. Although piperitenone does not constitute a major ingredient, it has also been isolated due to the fact that it is structurally similar to pulegone and piperitone.

Larvicidal Assays

Essential oils and their major ingredients were tested for their larvicidal activity against *Cx. pipiens* 3rd-4th instar larvae. EOpul1 was the most active essential oil as well as its major component pulegone among the essential oil major ingredients (based on non-overlapping confidence intervals) (see Table 2 for LC_{50} and LC_{90} values). The essential oils EOpul2 and EOsp and their dominated compounds, piperitone and carvone respectively, presented a rather medium activity (Table 2). EOpul3 was the only inactive essential oil showing no larvicidal effect. The larvicidal pattern of the EOpip was quite different: LC values were high and almost the same with the EOpul1 (LC_{50} value 40.28 and 46.97 mgl^{-1}

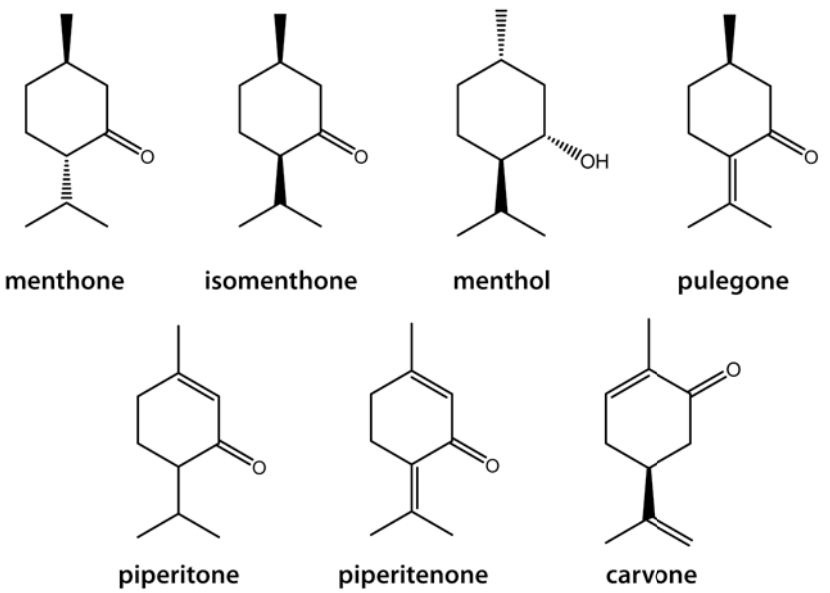

Figure 1. Chemical structures of essential oils' isolated components.

for EOpip and EOpul1 respectively).

Discussion

In this study, essential oils of *Mentha* species and their major ingredients were tested with respect to their larvicidal effect against *Cx. pipiens* mosquitoes. The selection of the three *Mentha* species, *M. pulegium* (three populations), *M. piperita* and *M. spicata*, was based on obtaining essential oils that possessed different chemical profiles. The isolated essential oils can be characterized as pulegone type (EOpul1), piperitone type (EOpul2), piperitone-isomenthone type (EOpul3), menthone-menthol type (EOpip) and carvone type (EOsp). These results of

Table 2. LC_{50} and LC_{90} values for essential oils and their isolated major components against larvae of *Culex pipiens* biotype *molestus*. Major components percentage composition, in relation to their precursor essential oils, is also given in parenthesis.

Essential oil / Major components (% composition in essential oil)	n[c]	Slope (±SE)	LC_{50} (95% CL)[a]	LC_{90} (95% CL)[a]	χ^2	df
EOpul1	400	4.93±0.53	46.97 (43.92-50.73)	85.42 (74.51-104.51)	15.440	18
pulegone (61.1%)	400	4.87±0.52	27.23 (24.39-29.7)	49.91 (45.4-56.61)	12.402	15
piperitenone (4.0%)	320	5.16±0.55	75.91 (72.54-79.2)	97.31 (92.16-104.84)	5.909	10
EOpul2	480	8.84±0.94	168.59 (157.76-182.99)	235.44 (209.67-293.82)	37.275[b]	16
Piperitone (92.6%)	320	8.47±0.9	131.91 (112.92-155.81)	186.84 (157.64-287.79)	47.024[b]	10
EOpul3	320	––	>200	––	––	––
piperitone (69.3%)	400	8.32±0.83	129.51 (118.78-142.17)	178.98 (159.16-223.33)	35.466[b]	13
isomenthone (24.8%)	320	––	> 200	––	––	––
EOsp	320	10.66±1.19	95.9 (80.44-107.13)	126.46 (112.57-162.87)	43.362[b]	10
carvone (71.8%)	400	5.54±0.68	95.31 (84.58-106.77)	162.31 (137.02-228.97)	26.735	13
EOpip	480	22.29±0.22	40.28 (30.9-50.56)	146.17 (105.4-252.54)	44.197[b]	19
menthone (39.0%)	480	9.29±0.92	111.11 (104.54-116.67)	152.63 (145.23-162.51)	18.942	16
menthol (25.9%)	400	8.6±1.31	120.97 (105.35-130.02)	170.45 (157.72-199.31)	20.977	13

[a] LC values are expressed in mgl^{-1} and they are considered significantly different when 95% CL fail to overlap.
[b] Since goodness–of–fit test is significant ($P<0.05$), a heterogeneity factor is used in the calculation of confidence limits (CL).
[c] Total number of larvae tested.

phytochemical analysis are in agreement with literature data (13, 17).

Essential oils are a mixture of different ingredients, mostly terpenes, which in the analysed *Mentha* spp. essential oils included pulegone, piperitone, isomenthone, carvone, menthone and menthol and they are comparable to findings in the literature (8, 13). Besides menthone, the rest isolated ingredients were evaluated for the first time against *Cx. pipiens* larvae. Among them, pulegone demonstrated the strongest larvicidal activity. The activity of all essential oils is in agreement with the proportion/toxicity rate of their individual major components, apart from the case of EOpip. The EOpip consists of menthone and menthol (in a total of 65%) and their LC_{50} values are higher than 100 mgl^{-1}, so it was expected to have the same toxicity pattern with the maternal essential oil. According to our experimental and literature data (10) EOpip did not follow this pattern and found to be one of the two most drastic essential oils (LC_{50} value near 40mgl^{-1}). Two suggestions could be made: either the rest of the ingredients possess independently strong larvicidal ability or some kind of synergistic phenomenon took place. These results are in accordance with those reported by Amer and Mehlhorn (2) where peppermint had moderate larvicidal activity against mosquito larvae (near 53% mortality after 24 h treatment).

Some interesting conclusions can be drawn concerning the relationship between the structure of the isolated *p*-menthane compounds and their larvicidal effect against *Cx. pipiens*. Although, pulegone and piperitone are isomers, the first is 5-fold more active than the later. This differentiation probably stands on account of the location of the C-C double bond that these two molecules contain. We assume that in pulegone the C-C double bond position on the chain group (isopropylidene *versus i*sopropyl group) enhances the toxicity. This hypothesis is strengthened by the case of piperitenone, where toxicity ranges between pulegone and piperitone. Piperitenone is structurally similar to both aforementioned molecules. Particularly, it combines all of the former molecules characteristics to one structure as it bares two double bonds, where the first one is internal at the cyclohexane ring (resembling to piperitone) and the second one external (resembling to pulegone). Therefore, two arguments can be made: either piperitenone's internal double bond reduces the total toxicity or its external double bond increases the toxicity. In any case, toxicity seems to be in direct connection to carbon-carbon double bond's position.

The effectiveness order of the above mentioned molecules, compared to menthone (saturated cetone), is the following: pulegone> piperitenone > menthone > piperitone. This ranking indicates that unsaturation might be a key factor but not necessarily the most determinant; it should be taken into account in combination with the position factor. Our results concerning carvone (a molecule isomer to piperitenone) also support this hypothesis: carvone is less effective than piperitenone while both of them are showing reduced toxicity compared to pulegone. This activity differentiation probably results from the different location of C-C double bond (isopropenyl group) as well as to its endo-exocyclic dienone character. By inference, it seems that in the case of the tested monoterpene cetones, the presence of the isopropylidene group increases the strength of larvicity, comparing to isopropenyl and/or isopropyl group effectiveness.

Finally, the use of the two enantiomers, isomenthone and menthone, revealed that the latter was more toxic, indicating that enantioselectivity may play also an important role for the toxicity of essential oils. This role of enantioselectivity has already been reported in previous projects and is a well-known fact: naphthoquinones (14), linalool (15) and limonene (16).

Essential oils have often proved to be more effective than their ingredients, indicating synergistic phenomena (9). Plants usually produce essential oils as a mixture of many ingredients with strong interactions among them. Results from this study

demonstrated that some *Mentha* species (*M. pulegium* and *M. piperita*) and their *p*-menthane type components (pulegone) could be potential larvicidal agents against *Cx. pipiens*. However, further investigations for their effects on non-target organisms, and their possible toxicity against mammals should be considered to ensure safety of application.

Literature Cited

1. Adams, R.P. 2007. *Identification of Essential Oils components by Gas Chromatography/Quadrupole Mass Spectroscopy, 4th Edition*; Allured Publishing Corp.: Carol Stream, Illinois.
2. Amer, A. and Mehlhorn, H. 2006. Larvicidal effects of various essential oils against *Aedes*, *Anopheles*, and *Culex* larvae (Diptera, Culicidae). *Parasitology Research,* 99: 466-472.
3. Ansari, M.A., Vasudevan, P., Tandon, M. and Razdan, R.K. 2000. Larvicidal and mosquito repellent action of peppermint (*Mentha piperita*) oil. *Bioresource Technology*, 71: 267-271.
4. Bakonyi, T., Hubálek, Z., Ivanics, E., Erdélyi, K., Ferenczi, E., Weissenböck, H. and Nowotny, N. 2008. In: Robinson WH, Bajomi D, eds. *Emergence of mosquito-borne flaviviruses in Central Europe*. Proceedings of the Sixth International Conference on Urban Pests. OOK-Press Kft. Hungary, p. 437-442.
5. Beereboom, J.J. 1966. The synthesis of piperitenone via mesityl oxide and methyl vinyl ketone. *Journal of American Chemical Society,* 31: 2026-2027.
6. CDC. Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases. http://www.cdc.gov/ncidod/dvbid/westnile/index.htm
7. Cheng, S.S., Huang, C.G., Chen, Y.J., Yu, J.J., Chen, W.J. and Chang, S.T. 2009. Chemical compositions and larvicidal activities of leaf essential oils from two eucalyptus species. *Bioresource Technology,* 100: 452-456.
8. De Andrade, I.L., Bezerra, J.S., Lima, M.A., De Faria, R.P., Lima, M.S. and Andrade-Neto, M. 2004. Chemical composition and insecticidal activity of essential oils from *Vallinosmopsis pohlii* baker against *Bemisia argentifolii*. *Journal of Agricultural and Food Chemistry,* 52: 5879-5881.
9. Don-Pedro, K.N. 1999. Investigation of single and joint fumigant insecticidal action of citrus peel oil components. *Pest Science,* 46: 79-84.
10. ECDC. European Centre for Disease Prevention and Control. http://www.ecdc.europa.eu. (see Events: Expert consultation on West Nile Virus infection in Europe. 25 Jan 2011 - 26 Jan 2011, Thessaloniki. Organized by: ECDC, Hellenic Center for Disease Prevention and Control (Greece), WHO Regional Office for Europe).
11. Floore, T.G. 2006. Mosquito larval control practices: past and present. *Journal of the American Mosquito Control Association,* 22: 527-533.
12. Kokkini, S., Hanlidou, E. and Karousou, R. 2004. Clinal variation of *Mentha pulegium* essential oils along the climatic gradient of Greece. *Journal of Essential Oil Research,* 16: 588-593.
13. Lupien, S., Karp, F., Wildung, M. and Croteua, R.B. 1999. Regiospesific cyrochrome P450 hydrolases from mint (*Mentha*) species: cDNA isolation, characterization, and functional expression of (-)-4*S*-limonene-3-hydroxylase and (-)-4*S*-limonene-6-hydroxylase. *Archives of Biochemistry and Biophysics*, 368: 181-192.
14. Melliou, E., Michaelakis, A., Koliopoulos, G., Skaltsounis, A. and Magiatis, P. 2009. High quality bergamot oil from Greece: chemical am=nalysis using enantiomeric GC-MS and larvicidal activity against the West Nile virus vector. *Molecules*, 14: 839-849.
15. Michaelakis, A., Koliopoulos, G., Stroggilos, A., Bouzas, E. and Couladouros E. 2009. Larvicidal activity of naturally occurring naphthoquinones and derivatives against the West Nile virus vector *Culex pipiens*. *Parasitology Research*, 104: 657-662.
16. Michaelakis, A., Koliopoulos, G., Milonas, P., Kontodimas, D., Polissiou, M., Kimbaris, A. and Papachristos, D. 2008. Activity of non-oxygenated versus oxygenated monoterpenes against mosquitoes. An attempt to correlate toxicity with chemical structure. In: A 7th Joint meeting of AFERP, GA, PSE & SIF. Natural products with pharmaceutical, nutraceutical, cosmetic and agochemical interest, Athens.
17. Miyazawa, M., Watanabe, H., Umemoto, K. and Kameoka, H. 1998. Inhibition of acetylcholinesterase activity by essential oils of *Mentha* species. *Journal of Agricultural and Food Chemistry,* 46: 3431-3434.
18. Papa, A., Danis, K., Baka, A., Bakas A, Dougas G, Lytras T, Theocharopoulos, G., Chrysagis, D., Vassiliadou, E, Kamaria, F., Liona A., Mellou, K., Saroglou, G. and Panagiotopoulos, T. 2010. Ongoing outbreak of West Nile virus infections in humans in Greece, July – August 2010. *Eurosurveillance*, 15: pii=19644 (Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19644)
19. Pitarokili, D., Michaelakis, A., Koliopoulos, G., Giatropoulos, A. and Tzakou, O. 2010. Chemical composition, larvicidal evaluation and adult repellency of endemic Greek Thymus essential oils against the mosquito vector of West Nile Virus.*Parasitology Research*, (DOI: 10.1007/s00436-011-2271-1).

20. Samarasekera, R., Weerasinghe, I.S. and Hemalal, K.D.P. 2008. Insecticidal activity of menthol derivatives against mosquitoes. *Pest Managment Science,* 64: 290-295.
21. Shaalan, E.A., Canyon, D.V., Younes, M.W.F., Abdel-Wahad, H. and Mansour, A.H. 2006. A review of botanical phytochemicals with mosquitocidal potential. *Environment International,* 31: 1149-1166.
22. Sukumar, K., Perich, M.J. and Boobar, L.R. 1991. Botanical derivatives in mosquito control: a review. *Journal of the American Mosquito Control Association,* 7: 210-237.
23. Trabousli, A.F., Taoubi, K., Samih, E.H., Bessiere, J.M. and Rammal, S. 2002. Incecticidal properties of essential oils against the mosquito *Culex pipiens molestus* (Diptera: Culicidae) *Pest Managment Science,* 58: 491-495.
24. WHO, *Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides*. Vol. WHO/VBC/81.807. 1981, Geneva: World Health Organization. p. 6.

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Τοξική δράση των αιθέριων ελαίων τριών ειδών *Mentha* spp. και των κυριότερων συστατικών τους στο κουνούπι *Culex pipiens*

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Περίληψη Η δράση των αιθέριων ελαίων από τρία διαφορετικά είδη *Mentha* spp. (Lamiaceae), καθώς και τα σημαντικότερα συστατικά τους, αξιολογήθηκαν έναντι προνυμφών του κουνουπιού *Culex pipiens* (Diptera: Culicidae). Ως εκ τούτου οι ουσίες πουλεγόνη, πιπεριτενόνη, πιπεριτόνη, καρβόνη, μενθόνη και μενθόλη απομονώθηκαν με χρωματογραφία στήλης. Τα αποτελέσματα έδειξαν ισχυρή δράση των αιθέριων ελαίων *M. pulegium* και *M. piperita* έναντι των προνυμφών (τιμές LD_{50} 46,97 και 40,28 mgl^{-1} αντίστοιχα) και η πουλεγόνη είχε την πιο ισχυρή δράση ανάμεσα στα κυριότερα συστατικά (27,23 mgl^{-1}). Για τα απομονωμένα μόρια, η διερεύνηση για πιθανή σχέση δομής δράσης αποκάλυψε ότι η θέση του διπλού δεσμού και η παρουσία της ισοπροπυλιδένο ομάδας μπορεί να είναι σημαντικοί παράγοντες δραστικότητας.

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First record and molecular identification of the parasitoid *Anagyrus* sp. near *pseudococci* Girault (Hymenoptera: Encyrtidae) in Greece - Host size preference for the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae)

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Summary The parasitoid *Anagyrus* sp. near *pseudococci* (Hymenoptera: Encyrtidae) was recorded for the first time in Greece and was identified using a PCR-based technique. Once identified, the host size preference of the parasitoid for the vine mealybug *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) was examined in choice experiments with four host size classes (0.5-0.9, 1-1.5, 1.6-2.3 and >2.3 mm) mostly consisting of one host stage (2nd, 3rd instar nymph, young female adult and preovipositing female adult, respectively). *Anagyrus* sp. near *pseudococci* preferably parasitized adult female mealybugs larger than 1.6 mm, whereas no successful parasitism was observed in smaller mealybugs (0.5-1.5 mm). The mean development time of the parasitoid at the two host size classes where parasitism occurred was 15.99 ± 0.43 days for females and 17.01 ± 0.53 days for males (28 ± 1°C, 16L:8D) and did not statistically differ between the sexes. Host size at parasitism did not affect the size of female and male parasitoid offspring.

Additional keywords: development, host selection, parasitism, vine mealybug

Introduction

The vine mealybug *P. ficus* is a pest of grapevine in the Medditerranean region, North and South Africa, Middle East, Mexico, California and Argentina. It is the most common mealybug species infesting grapevine in Greece (Heraklio); it has been a key pest on grapevine in South Africa for more than seventy years and it is a pest of economic importance damaging table and wine grapes as well as raisins in California (6, 10, 28, 30, 31, 33). Furthermore, *P. ficus* is a vector of the Grapevine Leafroll Associated Virus 3 (GLRa V-3) (13) so it is considered economically important even at low densities (15).

Chemical control of *P. ficus* is difficult and ineffective as the mealybug feeds on all parts of the grapevine (roots, trunk, cordons, canes, leaves, fruit) and a portion of its population often resides in protected locations i.e. under the bark of the trunk or cordon (8, 11, 15, 34). Biological control through introducing and/or fostering populations of natural enemies has provided an alternative method to suppress vine mealybug populations (7, 12, 34). The parasitoid *Anagyrus pseudococci* (Girault) (Hymentopeta: Encyrtidae) is the most commonly reared species among the natural enemies of the vine mealybug *P. ficus* (23) as usual with many *Planococcus* spp. and *Pseudococcus* spp. (31). Similarity in favourable climatic conditions and geographic distribution between *A. pseudococci* and the vine melybug renders it one of the most important biological agents

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of the pest (15, 16).

Anagyrus pseudococci is reported as a native endoparasitoid in many countries including, Argentina, Israel, Italy, South Africa and Turkey bu Gülek *et al.* (15) but morphological and molecular studies as well as cross-breeding experiments in cultures different origin which were originally considered as *P. pseudococci* revealed two distinct taxonomic forms: *Anagyrus* sp. near *pseudococci* and *A. pseudococci* (31).

Anagyrus sp. near *pseudococci* is a mainly Palearctic species, common in the Meditterranean region, which has also been established after introduction in California (31). *Anagyrus pseudococci* is considered aslo of Palearctic origin, believed to have been inintentioanally introduced in Argentina with seedlings from Italy (31). Both species coexist in Sicily. *Anagyrus* sp. near *pseudococci* resembles with *A. pseudococci* and the only morphological feature that discriminates them is found in the antennae: the first antennal funicle segment of female is partially black (basal half or so) and white (distal half or so) in *A. pseudococci* whereas it is entirely black in *A.* sp. near *pseudococci* (31). However, it is generally admitted that the subtle morphological differences between *A. pseudococci* and *A. sp.* near *pseudococci*, can sometimes be misleading (Noyes, personal communication), and thus only an expert could distinguish between them. DNA barcoding, based on a sequence from the Cytochrome Oxidase One (COXI) mitochondrial gene, has assisted in the resolution of various biological queries, ranging from detection of intraspecific variation to cryptic species recognition (4, 5, 26, 27, 28) and therefore could apply for the genetic identification of *A. sp.* near *pseudococci.*

Host preference has been defined as the relative frequency of host types chosen for parasitism compared with the frequency of host types available (18). Host stage or host size selection behaviour within a patch may be related to several factors such as differences in the profitability of each developmental stage e.g. small hosts may not provide adequate amounts of resource to support the successful development of parasitoid offspring (idiobionts) or can only produce small parasitoids in size which consequently reduces components of fitness such as longevity, fecundity and searching efficiency (idiobionts and koinobionts) (1). Small host size is often associated with lengthened development and/or reduced survival (14).

In the present work, the incidenece of the parasitoid *A.* sp. near *pseudococci* was proved from genetically identified samples in Greece. Moreover, the host size preference for the vine mealybug *P. ficus* and the effect of host size on development time and size of the parasitoid offspring were investigated as indices of the parasitoid fitness.

Materials and methods

Anagyrus sp. near *pseudococci* identification

The parasitoid samples were collected as parasitized mealybugs on *Mentha* spp. (Lamiaceae) at the region of Kifissia- Athens, Greece. The parasitoid species was first identified as *Anagyrus* sp., presumably *A.* sp. near *pseudococci versus Anagyrus pseudococci* (Girault) (Karamaouna, personal communication) based on morphological features (24, 31). Samples of both sexes of the parasitoid were sent to Dr John Noyes (Natural History Museum in the U.K.) for the systematic taxonomy of the species and they were identified as *Anagyrus* sp. of the pseudococci complex, which includes both *A. dactylopii* and *A. pseudococci*, sensu stricto and the so-called "Israeli" strain (Noyes, personal communication).

Molecular techniques were used for the verification of the sampled species. Insect DNA was extracted using the GenEluteTM 100 Kit (Sigma) following the protocol of the manufacturer. An amplicon of about 900 bp from the mitochondrial COI gene was polymerised in 25μl reactions containing 1.5 mM MgCl2, 50 M dNTPs, 0.2 _M of the primers MTDNA04 (forward) (AGGAAC(AT)GG(AG) TGAACNGTTTA(TC)CC(AT)CC) (Avtzis, Navrozidis and Tsoktourides, unpublished data) and UEA10 (reverse) (22), and 1U of Taq poly-

merase. PCR was performed with a thermocycler (Primus 25, PeqLab) with the following amplification conditions: initial denaturation phase at 97°C for 4 min, 35 cycles of denaturation at 96°C for 25 s, annealing at 48°C for 25 s and extension at 68°C for 90 s were followed by a final extension step at 68°C for 10 min. PCR products were purified using the QIAquickTM 108 Kit (QiaGen) and directly sequenced with both primers described above on an ABI 3770 capillary sequencer (Applied Biosystems), in order to exclude cases of base misincorporation due to PCR error (20) and visualised using CHROMAS LITE (37).

The 818 bp long sequences were aligned by eye and were put into the GenBank (36). There, the similar sequences were determined using the Basic Local Allignment Search Tool (BLAST) by nucleotide. In addition, twelve nucleotide sequences of three *Anagyrus* species already submitted to GenBank were used in the construction of a phylogenetic tree. Specifically, *Anagyrus pseudococci* (DQ667743, DQ667745, DQ667744, DQ667746, DQ667747) *Anagyrus sp.* near *pseudococci* (DQ667737, DQ667738, DQ667739,DQ667740,DQ667741,DQ667742) and *Anagyrus dactylopii* (DQ667736) sequences were used. A Neighbor-Joining (NJ) approach (25) was applied to construct a tree from the pairwise distances that were estimated using the substitution model of Tamura and Nei (29) as implemented in MEGA version 3.1 (21).

Bioassay

The primary culture of the mealybug *P. ficus* was established in the insectary of the Biological Control of Pesticides Laboratory at Benaki Phytopathological Institute from individuals which were collected in an infested vineyard at the region of Helia-Peloponnese. Taxonomic identification of the species was done according to Cox and Ben-Dov (6) key (P. Milonas and F. Karamaouna, personal communication). The culture was maintained on sprouted potatoes of the variety 'Marfona' in sandwich boxes (17x11x5 cm: Length x Width x Height) with net covered openings (d= 1.5 cm) for ventilation. The boxes were kept in a Gallenkamp CO_2 growth chamber at 26°C and constant dark. All biological stages of the mealybug were present in the culture.

The parasitoid colony was reared on sprouted potatoes from the *P. ficus* mass culture in plexiglass cages [50x40x40 cm with two net covered ventilation openings (30x20 cm)] in the insectary at 28 ± 1°C and L16:D8 h. In order to schedule parasitoid emergence for the experiment, parasitoids of both sexes from the colony were released in sandwich boxes (5-10 individuals/box) and were let to parasitise mealybugs on the infested potatoes every week.

Individuals of the pest were transferred from the mass culture on leaf sections (central part) of *Nerium oleander* (Apocynaceae), which is a host plant of the mealybug (Scalenet database), a few hours before the experiment. The leaf sections were kept with their lower surface upwards on top of a layer of 8 g/l Agar, which had previously been autoclaved, in Petri-dishes of 9 cm diameter. Size classes of the host were used rather than host stages but they were selected so that each size class comprised mostly one stage according to sampled sizes of mealybugs measured after moults. The size classes were 0.5-0.9 mm (2nd instar nymph), 1-1.5 mm (3rd instar nymph), 1.6-2.3 mm (young female adult), >2.3 mm (preovipositing female adult) [Classification by Karamaouna and Copland (21)]. Only female mealybugs were used in the experiments apart from the two small size classes where males and females could not be distinguished. Five mealybugs of each size class (20 in total) were placed randomly in each Petri dish.

Every 1-3 days, females of *A.* sp. near *pseudococci* were collected from the sandwich boxes of the mass parasitoid culture, in which both female and male wasps were present. These 1-3 days old females were assumed to have mated and were used in the experiments. During the experiments, wasps were released individually in the Petri-dishes for 24 hours. At the end of the experiment, the ovipositing female wasps were measured (head-width) and dissected to confirm that they had been inseminated (17). The Petri-dishes were

kept in a growth room at 28 ± 1°C and L16:D8 h until the mealybugs became mummified. The mummies were collected, measured (tip of the head to the end of the abdomen) and kept individually in 0.5 ml Eppendorf tubes, under the aforementioned conditions of temperature and light until parasitoid emergence. A binocular stereo-microscope (X 5 – X 50) with a linear scale graticule fitted in one of its lenses was used for all the necessary measurements of the host and the parasitoid.

Proportion of mealybugs mummified out of the total number in each size class of the host (index of host size preference) as well as the sex, development time and size (headwidth) of the parasitoid offspring were recorded (body size is usually a good predictor of fecundity in parasitoids).

Data analysis

The four host size classes, offered simultaneously to the parasitoid in each Petri dish, were the treatments. Each Petri-dish was considered as one experimental unit – replication of the experiment. Fifty experimental units were set but parasitism was observed only in 34 Petri dishes in the two larger classes of the host. Because observations of host size classes within the same experimental unit were not independent, the 34 observations were divided into two groups and data on development time and headwidth of one parasitized host size class from one group were compared with the respective data of the other size class from the second group using ANOVA (α = 0.05) in MINITAB (Release 10.51 Xtra). Data on parasitism rate were subjected to angular transformation to fulfill the assumptions of normality and homogeneity of variance before ANOVA.

Results

Molecular identification of *A.* sp.near *pseudococci*

The employment of a PCR-based method in the identification of the *Anagyrus* species, through either BLAST or phylogenetic tree contruction yielded a similar outcome. BLAST indicated a high similarity of the 818 bp long sequence with *Anagyrus* sp. near *pseudococci* sequences, whereas it was less similar with the sequences of *A. pseudococci* and *A. dactylopii*. Furthermore, the distance-based calculation of the phylogenetic tree clustered the sequences in the clade of the *A.* sp.near *pseudococci* sequences obtained from the NCBI GeneBank, supporting this clade with high bootstrap value (Bootstrap Value: 100%) whereas sequences from *A. pseudococci* individuals were separately clustered into another distinct monophyletic clade (Figure 1).

Host size preference of the parasitoid *A.* sp.near *pseudococci*

The evaluation of host size preference was based on the rate of parasitized mealybugs (mummies) in each size class. No parasitism was observed in the smaller host size classes (0.5-0.9 mm and 1-1.5 mm). The rate of parasitism in the larger size classes was 0.18-0.22 and did not statistically differ between them. (ANOVA, $F_{1, 48}$ = 0,37, P= 0,544) (Table 1).

Effect of host size of *P. ficus* on development time and size of the parasitoid offspring *A.* sp. near *pseudococci*

The mean development time of female and male offspring of *A.* sp. near *pseudococci* in female adults did not significantly differ between the size classes '1.6-2.3 mm' and '>2.3 mm' (females: ANOVA, $F_{1,15}$ = 0.02, P= 0.877; males: ANOVA, $F_{1,20}$ = 0.94, P= 0.344) (Table 2). No significant difference was found in development time of female and male parasitoids, when the pooled data of the two size classes were analysed (ANOVA, $F_{1, 37}$ = 2.06, P= 0.160) (Table 2).

The mean size (headwidth) of female and male offspring of the parasitoid *A.* sp. near *pseudococci* in female adults (1.6-2.3 mm and >2.3 mm) of *P. ficus* did not significantly differ significantly between the size classes (females: ANOVA, $F_{1,10}$ = 0.92, P= 0.360; males: $F_{1,30}$ = 0.01, P= 0.932) (Table 2). Female parasitoids were larger (headwidth) than male parasitoids based on the pooled

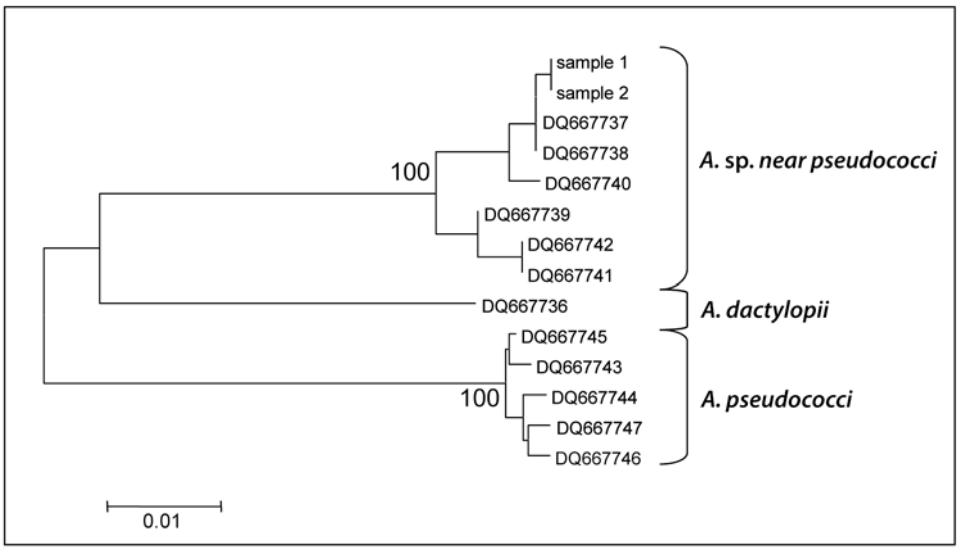

Figure 1. Neighbor-Joining phylogenetic tree of the samples and the sequences retrieved from NCBI databank based on the Tamura-Nei nucleotide substitution model (bootstrap repeats = 500). The bar indicates a TN genetic distance of 0.01.

Table 1. Rate of *P. ficus* mealybugs parasitized by *A.* sp. *near pseudococci* when different host size classes were simultaleously offered to the parasitoid for 24 hours.

Host size classes (mm)	Rate of parasitism	
	n	$\bar{x}$ ± s.e.
1.6–2.3	25	0.22 ± 0.05
>2.3	25	0.18 ± 0.05

n: number of replications

Table 2. Development time and size (headwidth) of female and male offspring of the parasitoid *A.* sp. near *pseudococci*, which emerged from different size classes of female adults of the mealybug *P. ficus*.

Host size classes (mm)	Parasitoid development time (days)				Headwidth (mm)			
	Females		Males		Females		Males	
	n	$\bar{x}$ ± s.e.	n	$\bar{x}$ ± s.e.	n	$\bar{x}$ ± s.e.	n	$\bar{x}$ ± s.e.
1.6–2.3	8	15.92 ± 0.66	10	17.58 ± 0.99	5	0.51 ± 0.03	19	0.45 ± 0.01
> 2.3	9	16.06 ± 0.59	12	16.54 ± 0.52	7	0.54 ± 0.02	13	0.46 ± 0.01
>1.6 (both size classes)	17	15.99 ± 0.43	22	17.01 ± 0.53	12	0.53 ± 0.02	21	0.46 ± 0.009

n: number of observations

data of both parasitized size classes (ANOVA, $F_{1,31}$ = 15.04, P= 0.001) (Table 2).

Size of male wasps was related positively to a straight line with the host size (Linear Regression ANOVA, $F_{1,30}$ = 11.79, P= 0.002) but the size of female wasps was not (ANOVA, $F_{1,10}$ = 3.56, P= 0.088); however the female sample was low. In addition the regression line coefficient (r^2) of the estimated regression line for males (headwidth = 0.276 + 0.016 x mumy size) was low (r^2 = 0.282).

Discussion

The parasitoid *Anagyrus* sp. near *pseudococci* was recorded for the first time in Greece and identified with accuracy using a PCR-based technique. Varikou *et al.* (33) have reported the presence of *A. pseudococci* in Crete and attributed effective biological control of *P. ficus* to the parasitoid in organic vineyards. In addition there is only one record of *Anagyrus* sp. on *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) in Greece (2).

The phylogenetic comparison of the sequences of *Anagyrus* sp. near *pseudococci* with those of *A. dactylopii* and *A. pseudococci* revealed that *A.* sp.near *pseudococci* is genetically closer to *A. dactylopii* than to *A. pseudococci,* an outcome fully congruent with the conclusion of Triapitsyn *et al.* (31).

Anagyrus sp. near *pseudococci* seems to prefer parasitizing female adults of the vine mealybug *P. ficus*, larger than 1.6 mm, whereas no successful parasitism is observed in smaller mealybugs (0.5-1.5 mm). Female adults (young and pre-ovipositing) appear to be the most profitable host stage of *P. ficus* for the development of *A.* near *pseudococci* based on host preference and successful parasitism. Laboratory experiments by Daane and Bendley (10) with the parasitoid *A. pseudococci* indicated also a host size preference for larger hosts of the mealybug *P. ficus* based on the parasitoid's attempts for oviposition in 2nd and 3rd instar nymphs and female adults but successful parasitism by *A. pseudococci* was not studied then. Gülec *et al.* (15) reported successful parasitism of 3rd instar nymphs and young female adults of *P. ficus* by *A. pseudococci* in no-choice experiments, indicating that even 3rd instar nymphs provide adequate resources for successful parasitoid development.

Parasitoid fitness, in particular shorter development time and larger size, of *Anagyrus* sp. near *pseudococci* was not directly related with the host size at parasitism. The development time of *Anagyrus* sp. near *pseudococci* in young and pre-ovipositing female adults of *P. ficus* was 16-17 days (for female and male parasitoids, respectively), which is comparable to the development time of 15.2-16.6 days (for male and female parasitoids, respectively) in *A. pseudococci* found by Gülec *et al.* (15) at the same mealybug species and similar temperature and photoperiod conditions (28 ± 1°C and 16L:8D). The host size at parasitism did not affect the size of offspring of *Anagyrus* sp. near *pseudococci* in either sex, which probably means that this is a koinobiont parasitoid like *A. pseudococci* (15).

Males and females of *Anagyrus* sp. near *pseudococci* took similar time to develop in our study whereas Gülec *et al.* (15) reported that males of *A. pseudococci* developed faster than females in young female adult mealybugs. However, female parasitoids emerging from hosts larger than 1.6 mm were larger than male parasitoids emerging from the same host size class as it usually occurs in solitary Hymenoperan parasitoids of other mealybug species or other insect pests (3, 19).

Should *A. pseudococci* and *Anagyrus* sp. near *pseudococci* be biologically separate species or not, the present preliminary host size preference bioassay supports that both of them have a preference for larger hosts of the vine mealybug *P. ficus*.

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Literature Cited

1. Alphen, J.J.M. van and Jervis, M.A. 1996. Foraging Behaviour. pp. 1-62. In: M. Jervis and N. Kidd (eds.) *Insect Natural Enemies: Practical Approaches to their Studies and Evolution,* Chapman and Hall, London.
2. Argyriou, L.C., Stavraki, H.G. and P.A. Mourikis 1976. A list of recorded entomophagous insects of Greece. Benaki Phytopahtological Institute, Kifissia-Athens, Greece.
3. Asante, S.K. and Danthanarayana, W. 1993. Sex ratios in natural populations of *Aphelinus mali* (Hym: Aphelinidae) in relation to host size and host density. *Entomophag*a, 38: 391-403.
4. Avise J.C. 2000. *Phylogeography - The history and formation of species*. Harvard University Press.
5. Avtzis, D.N., Arthofer, W. and Stauffer, C. 2008. Sympatric occurrence of diverged mtDNA lineages of *Pityogenes chalcographus* (Coleoptera, Scolytinae) in Europe. *Biological Journal of the Linnean Society,* 94: 331–340.
6. Ben-Dov, Y., Miller, D.R. and Gibson, G.A.P. (2011) Scalenet: A datadase of the scale insects of the world <htpp:www.sel.barc.usda.gov/scalenet/scalenet> (4-7-2011).
7. Berlinger, M. 1977. The Mediterranean vine mealybug and its natural enemies in southern Israel. *Phytoparasitica*, 5: 3–14.
8. Castillo A.A.F., Hernández, H.G. and Daane K.M. 2005. *Los Pijo Harinosos de la Vid*. Libro Técnico No. 9. Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Sonara, Mexico.
9. Cox, J.M. and Ben-Dov, Y. 1986. Planococcine mealybugs from the Mediterranean Basin and their distinction from a new African genus (Hemiptera: Pseudococcidae) *Bulletin of Entomological Research*, 76: 481-489.
10. Daane, K.M. and Bentley, W.J. 2000. University of California Cooperative Extension <http://vinemealybug.uckac.edu>
11. Daane K.M., Malakar-Kuenen, R., Guillén, M., Bentlyey, W.J., Bianchi, M. and D. Gonzalez, D. 2003. Abiotic and biotic refuges hamper biological control of mealybug pests in California vineyards. *Proceedings of the 1st International Symposium on Biological Control of Arthropods* (ed. R. van Driesch), pp. 389–398. FHTET-03055. USDA Forest Service Publishers, Morgantown, West Virginia.
12. Duso, C. 1990 (1989). Indagini bioecologiche su *Planococcus ficus* (Sign.) nel Veneto. *Bollettino del Laboratorio di Entomologia Agraria "Filippo Silvestri"*, 46: 3–20.
13. Engelbrecht, D. and Kasdorf, G. 1990. Transmission of grapevine leafroll disease and assocaited closteroviruses by the vine mealybug, *Planococcus ficus*. *Phytophylactica*, 22: 341–346.
14. Godfray, H.C.J. 1994. Parasitoids: Behavior and Evolutionary Ecology. Princeton University Press, Princeton, NJ, USA.
15. Gülec, G., Kilincer, A.N., Kaydan, M.B. and Ulgenturk, S. 2007. Some biological interactions between the parasitoid *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae) and its host *Planococcus ficus* (Signoret) (Hemiptera: Coccoidea: Pseudococcidae). *Journal of Pest Science,* 80: 43-49.
16. Gutierez, A.P., Daane, K.M., Ponti, L., Walton V.M. and Ken Ellis C. 2008. Prospective evaluaton of the biological control of the vine mealybug: refure effects and climate. *Journal of Applied Ecology*, 45: 524-536.
17. Jong de, P.W., and van Alphen, J.J.M. 1989. Host size selection and sex allocation in *Leptomastix dactylopii*, a parasitoid of *Planococcus citri*. *Entomologia Experimentalis et Applicata*, 50: 161-169.
18. Hopper, K.R. and King, E.G. 1984. Preference of *Microplitis croceipes* (Hymenoptera: Braconidae) for instars and species of *Heliothis* (Lepidoptera: Noctuidae). *Environmental Entomology,* 31: 1145-1150.
19. Karamaouna, F. and Copland, M.J.W. 2000 Host suitability, quality and host size preference of *Leptomastix epona* and *Pseudaphycus flavidulus*, two endoparasitoids of the mealybug *Pseudococcus viburni*, and host size effect on parasitoid sex ratio and clutch size. *Entomologia Experimentalis et Applicata* 96: 149-158.
20. Kobayashi, N., Tamura, K. and Aotsuka, T. 1999. PCR error and molecular population genetics. *Biochemical Genetics:* 37, 317-321.
21. Kumar, S., Tamura, K. and Nei, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics,* 5: 150-163.
22. Lunt, D.H., Zhang, D.-X., Szymura, J.M. and Hewitt, G.M. 1996. The cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. *Insect Molecular Biology*, 5: 153-165.
23. Malakar-Kuenen, R., Daane, K.M., Bentley, W.J., Yokota, G.Y., Martin, L.A., Godfrey, K. and Ball, J. 2001. Population dynamics of the vine mealybug and its natural enemies in the Coachella and San Joaquin Valleys. *University of California Plant Protection Quarterly*,11: 1–3.
24. Rosen, D. and Rössler. Y. 1966. Studies on an Israel strain of *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae). I. Morphology of the adults and developmental stages. *Entomophaga,* 11: 269-277.
25. Saitu, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4: 406-425.
26. Smith, M.A., Woodley, N.E., Janzen, D.H., Hallwachs, W. and Hebert, P.D. 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proc. Natl. Acad. Sci. U.S.A,* 103: 3657–62.

27. Smith, M.A., Wood, D.M., Janzen, D.H., Hallwachs, W. and Hebert, P.D. 2007. DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera: Tachinidae) are not all generalists. *Proc. Natl. Acad. Sci. U.S.A.,* 104: 4967–4972.
28. Stauffer, C., Riegler, M. and Lakatos, F. 2001. *Ips typographus f. japonicus* Niijima (Coleotpera, Scolytidae): A genetic analysis by allozymes and mitochondrial sequence data. In: Jandl R, Devall M, Khorchidi M, Schimpf E, Wolfrum G & Krishnapillay B (eds.). *Forests and Society: The Role of Research*. XXI IUFRO World Congress 7-12 August 2000. Kuala Lumpur, Malaysia: Pramaju Sdn. Bhd, Vol. 3, 397.
29. Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of the mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, 10: 512-526.
30. Tranfaglia, A. 1981. Les pseudococcines de la vigne. Atti Terzo Incontro "La Difesa Integrata della Vite" Latina, 3-4 Dec. 1981, pp. 211-215.
31. Triapitsyn, S.V., Gonzalez, D., Vickerman, D.B., Noyes, J.S. and White, E.B. 2007. Morphological, Biological and molecular comparisons among the doifferent geographical populations of *Anagyrus pseudococci* (Hymenoptera: Encytridae), parasitoids of *Planococcus* spp. (Hemiptera: Pseudococcidae), with notes on *Anagyrus dactylopii*. *Biological Control*, 41: 14-24.
32. Tzanakakis, M.E. and Katsoyannos B.I. 2003. Insects of fruit trees and grapevine. Agrotypos, Marousi-Greece, p. 360 (in greek).
33. Varikou, K., Birouraki, A., Bagis, N. and Kontodimas, D.C. 2010. Effect of temperature on the development and longevity of *Planococcus ficus* (Hemiptera: Pseudococcidae). *Annals of Entomological Society of America,* 103: 943-948.
34. Walton, V.M. 2003. Development of an integrated pest management system for vine mealybug, *Planococcus ficus* (Signoret), in vineyards in the western CapeProvince, South Africa. PhD Thesis. University of Stellenbosch, Stellenbosch, South Africa.
35. Walton, V.M and Pringle K.L. 2005. Developmental biology of vine mealybug, *Planococcus ficus* (Signoret) (Homoptera: Pseudococcidae), and its parasitoid *Coccidoxenoides perminutus* (Timberlake) (Hymenoptera: Encyrtidae). *African Entomology,* 13: 143-147.

Web sites

36. http://www.ncbi.nlm.nih.gov/
37. http://www.technelysium.com.au

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Πρώτη καταγραφή και μοριακή ταυτοποίηση του παρασιτοειδούς *Anagyrus* sp. near *pseudococci* Girault (Hymenoptera: Encyrtidae) στην Ελλάδα – Προτίμηση ως προς το μέγεθος του ψευδόκοκκου-ξενιστή *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae)

Φ. Καραμαούνα, Γ. Μενούνου, Γ.Ι. Σταθάς και Δ.Ν. Αβτζής

Περίληψη Το παρασιτοειδές *Anagyrus* sp. near *pseudococci* (Hymenoptera: Encyrtidae) καταγράφηκε για πρώτη φορά στην Ελλάδα και ταυτοποιήθηκε με την βοήθεια τεχνικών PCR. Επίσης εξετάστηκε η προτίμηση του παρασιτοειδούς ως προς το μέγεθος του ψευδόκοκκου-ξενιστή *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) σε εργαστηριακά πειράματα όπου ήταν διαθέσιμες τέσσερις κλάσεις μεγέθους (0.5-0.9, 1-1.5, 1.6-2.3 and >2.3 mm) οι οποίες αντιστοιχούν κυρίως σε ένα βιολογικό στάδιο του εντόμου (νύμφες $2^{ης}$ και $3^{ης}$ ηλικίας, νεαρά ακμαία θηλυκά και ακμαία θηλυκά πριν την ωοτοκία, αντίστοιχα). Το *Anagyrus* sp. near *pseudococci* παρασίτησε κατά προτίμηση ακμαία θηλυκά άτομα του ψευδόκοκκου μεγαλύτερα από 1.6 mm, ενώ δεν παρατηρήθηκε επιτυχής παρασιτισμός σε μικρότερα άτομα (0.5-1.5 mm). Η μέση διάρκεια ανάπτυξης του παρασιτοειδούς στις δύο κλάσεις μεγέθους του *P. ficus* που παρατηρήθηκε παρασιτισμός ήταν 15.99 ± 0.43 ημέρες για τα θηλυκά άτομα και 17.01 ± 0.53 ημέρες για τα αρσενικά (28 ± 1°C, 16L:8D) χωρίς στατιστικά σημαντική διαφορά μεταξύ των δύο φύλων. Το μέγεθος του ξενιστή κατά τον παρασιτισμό δεν είχε επίδραση στο μέγεθος των απογόνων του παρασιτοειδούς.

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SHORT COMMUNICATION

First record of *Lixus umbellatarum* Fabricius (Coleoptera: Curculionidae) in Greece, on the medicinal herb *Opopanax chironium*

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Summary In summer 2004, adults of a Coleopteran species were observed piercing with their rostrum the stems of the plant *Opopanax chironium* at an altitude of 1850 m on Parnassus Mountain. Upon insect's feeding, the effused sap of the plant turned into droplets of a resinous fluid which is known to have pharmaceutical and other useful properties. The insect was identified as *Lixus umbellatarum* Fabricius of the Curculionidae family and it is recorded for the first time in Greece.

Additional keywords: Apiaceae, Coleoptera, Greece, Panacea, Panax, Umbelliferae

In summer 2004, *Opopanax* sp. plants were observed on the northwest (NW) side of Mount Parnassus, Greece to bare light-orange coloured droplets of a resinous liquid on their stems lengthwise, in a rather straight line and regular distance between them. Because of the late season, the plants carried only mature umbels; therefore proper identification of the taxon was not possible. In May 2006, the droplets observed on the *Opopanax* sp. plants were associated with an adult weevil, which was found at the same site to feed on the stem, causing effusion of the sap (Fig.1). At that time, a plant specimen was collected in order to form a proper herbarium for its definite identification. After the ordinary procedure, which included drying and disinfestations at low temperatures, the plant sample was stored among the other Apiaceae (Umbelliferae) collections of the Agricultural University of Athens (AUA). One year later, the plant sample was found to be totally devoured by the recorded weevil which had been accidentally introduced into the sample and survived in -22° C for about a two month period. In mid April of 2008, when the *Opopanax*

Figure 1. *Lixus umbellatarum* feeding on *Opopanax chironium* stem.

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sp. plants possess only their lower leaf rosettes and the basal part of the previous year's stem, living weevil adults were found in the openings of exit holes at the base of the dead stems, indicating that the insect had survived the severe winter conditions at 1850 m. altitude. In summer of 2008, several individuals of the weevil were collected and identified in the Laboratory of Entomology of AUA.

All biological material was collected in the NW side of the Mountain Parnassos – Greece at the location "Kokinorachi" (latitude 38 34΄ N., longitude 22 28΄ E., altitude 1850 m), in the clearings of an *Abies cephallonica* forest with limestone substrate, in consequent years from 2004 to 2008.

Plant material presented a distinct and unique combination of characters attributed to genus *Opopanax* W.D.J. Koch. (3, 7, 16, 17). These are the excessive presence of stellate hairs in the stem and the leaves, the large 2-pinnate basal leaves with ovate leaf-lobes, the yellow petals and the strongly compressed obovate fruit (17). *Opopanax* W.D.J. Koch consists of stout perennial herbs, including 3 species worldwide (13). The species which occur in Greece include *O. hispidus* (Friv.) Griseb. and *O. chironium* (L.) Koch (17) but the presence of the later above 1.500 m is dubious (16).

The herbarium identification was based on the four most prominent identification keys of these species (3, 7, 16, 17). The original key character is related to the size and the border of the fruit, which in the examined herbarium was 6-7 mm with a narrow and thickened border, concluding to *O. chironium* (7, 17). Later key characters advocating this identification are the rays' number, which in the herbarium was counted up to 23 (3) and the leaf lobes length which was measured up to 14 cm (16, 17). A deviation from the described key characters was observed in relation with the thickened border's width, which was measured from 1 to 2 mm, when in *O. chironium* is described less than 1 mm, and in *O. hispidus* between 2-3 (3, 16). This deviation can be explained by the geographical (16) or territorial (3) specialization of the related keys, the first one referring to the mountain flora of Greece and the second to Turkey and East Aegean. Another inquiring character, observed in the specimen and was never described before, relates to the presence of reticulate, ribbed and brightly yellow coloured veins both in basal and cauline leaves. This secondary character, absent in the population of *O. chironium* from Peloponnesus and the island of Rhodes, which was not infected by the insect, could be considered as a response to the insect's presence. However, this hypothesis needs further experimental confirmation.

The identification of the insect to the species was made by the first author (specialist on Curculionidae) based on classical monographs (9, 14, 15). The genus *Lixus* is characterized by a cylindrical rostrum and elytra rather narrow and oblong ending in two separate points. The body surface seems to be covered with yellow or reddish 'dust' (efflorescence) while its basic colour is black with dense and thin punctuation and the abdominal pubescence is yellowish with bi- or trifide setae. *Lixus* (*Eulixus*) *umbellatarum* belongs to Rhynchophora Coleoptera, the subfamily Cleoninae and the tribe Lixini, with the following taxonomic history: *Lixus (Eulixus) umbellatarum* Fabricius, 1787 primary genus: *Curculio,* subgenus *Eulixus* Reitter, 1916 = *Promecaspis* Hoffmann, 1958. species: *pardalis* Boheman, 1835 –*submaculatus* Boheman, 1842 –*brevicaudatus* Lucas, 1848 –*brevicaudis* Küster, 1849. Species' characteristics include: Body length 8-14 mm. Efflorescence of pronotum, greenish-yellow. Rostrum of female slender, cylindrical, convex, shinny, longer than the prothorax. Rostrum of male less convex, opaque, densely punctuated. Antennae short, fairly slender with the first two segments of the flagellum equal, the 3rd to 6th being of the same diameter and the 7th larger. Antennal club ellipsoid and sharp- pointed. Antennae (except the club) and tarsi, ferruginous. Eyes oval and convex. Prothorax conical, more slender in the male. Elytra, at their base, slightly larger than prothorax. Tarsi short (Hoffmann, Por-

ta, Portevin).

The presence of *L. umbellatarum* has been confirmed in France (Corsica) (14, 15) and in Italy (Latsio, Campania, Pulia, Sardinia) (14). According to Hoffman (9), the insect is rare in the French mainland but abundant in Corsica, feeding on miscellaneous Apiaceae such as *Ferula communis* L., *Pastinaca lutea* L. and *O. chironium*. It is also found in Spain, Portugal, Algeria and Morocco. There are 'no data' for its presence in the Greek mainland and the islands (19).

The host plants of *L. umbellatarum* belong to the families Apiaceae, Chenopodiaceae, Polygonaceae, Caryophylaceae, Brassicaceae (Cruciferae), Lamiaceae (Labiatae), Papilionaceae, Capparidae, Malvaceae and Geraniaceae. The larvae of the weevil feed on the internodes of the stem; they bore straight descending galleries through petioles and stems attaining the plant's 'neck' or the upper part of the roots. (12). Hibernation takes place in the adult stage, usually in the soil, appearing during March and April and feeding up to September. Pupation takes place at the end of the galleries and life cycle usually lasts one year.

The present report associates feeding of the weevil *L. umbellatarum* on *O. chironium* with the produce of resin by the plant. *Opopanax chironium* is richly celebrated through history for the medicinal properties of its resin, Panacea, applied mainly as analgesic and venom's antidote (4). Among the natural products that have been identified in *O. chironium* are C-17 acetylenes (2), various phthalides in the roots (6), coumarin derivatives (1, 8) and irregular diterpenoids (11), while crude extracts of the plant present significant apoptotic activity (1). *Opopanax* sp. resin and oil are also used as a direct food additive (18), expectorant and antispasmodic (5, 11) and in perfumery (5). Despite this extensive use of *Opopanax* sp. resin, the presence of *L. umbellatarum* has never been reported before as a stimulating agent that induces resin production. This suggestion is advocated by the fact that several of its compounds, such as coumarins, furanocoumarins and flavonoids, are identified as constitutive antifungal agents or phytoalexins (10). Experimental investigation is required in order to ascertain the recorded insect-plant interaction and determine the role of the weevil in the induction of Panacea.

Literature cited

1. Appendino, G., Bianchi, F., Bader, A., Campagnuolo, C., Fattorusso, E., Taglialatela-Scafati, O., Blanco-Molina, M., Macho, A., Fiebich, B.L., Bremner, P., Heinrich, M., Ballero, M. and Munoz, E. 2004. Coumarins from Opopanax chironium. New Dihydrofuranocoumarins and Differential Induction of Apoptosis by Imperatorin and Heraclenin. *Journal of Natural Products*, 67: 532-536.
2. Bohlmann, F. 1971, Acetylenic compounds in the Umbelliferae, *Botanical Journal of the Linnean Society*, 64: 279-291.
3. Davis, P.H. 1984. *Flora of the Turkey and the East Aegean islands*, Edinburg University Press, vol. 4, pp 471-473.
4. Dioscorides, 1st-2nd cent. A.D., De Materia Medica, *Codex Neapolitanus Graecus 1 of the National Library of Naples*, Photographic reprint by Genus Publications, Athens, Greece, 2001.
5. French D.H., 1971, Ethnobotany of the Umbelliferae, *Botanical Journal of the Linnean Society*, 64: 385-412.
6. Gijbels, M.J.M., Bos, R., Scheffer, J.J.C. and Svendsen, A.B. 1983. Phthalides in roots *Opopanax chironium*, *Planta Medica*, 47: 3-6.
7. Halacsy, E. 1900. *Conspectus Florae Graecae*, Lipsiae Sumptibus Guilelmi Engelmann, vol. 1, p 637.
8. Hegnauer R., 1971, Chemical patterns and relationships of Umbelliferae, *Botanical Journal of the Linnean Society*, 64: 267-277.
9. Hoffmann, A. 1950. *Faune de France, 52 (2ème partie): Curculionidae*, Librairie de la Faculté des Sciences, Paris.
10. Harborne, J.B. and Williams, C.A. 2000. Advances in Flavonoid research since 1992. *Phytochemistry*, 55: 481-504.
11. Muckenstrum, B., Foechterlen, D., Reduron, J.-P., Danton, P. and Hildenbrand, M. 1997. Phytochemical and chemotaxonomic studies of *Foeniculum vulgare*. *Biochemical Systematics and Ecology*, 25: 353-358.
12. Perrier, R. 1932. *La Faune de France, Fasc.6: Coléoptères*, Librairie Delagrave, Paris.
13. Pimenov, M.G., Leonov, M.V. 1993. *The genera of the Umbelliferae*. Royal Botanic Gardens Kew & Botanical Garden of Moscow State University, Kent U.K., pp 156.
14. Porta, A. 1932. *Fauna Coleopterorum Italica, Vol.*

V: Rhynchophora – Lamellicornia, Stab. Tipogr. Piacentino, Piacenca.

15. Portevin, G. 1935. *Histoire Naturelle des Coléoptères de France, Tome IV: Polyphaga – Rhynchophora*, Paul Lechevalier, Paris.
16. Strid, A. 1986. *The mountain flora of Greece*, Cambridge University Press, vol. 1, p 714.
17. Tutin, G.T., Heywood, H.V., Burges, A.N., Moore, M.D., Valentine, H.D., Walters, M.S. and Webb, A.D. 1972. *Flora Europaea*, Cambridge University Press, vol. 3, p 360.
18. United States of America. 1966. *Legislation Regulations governing the use of colour additives, pesticides and food additives*, Fd Cosmet. Toxicol Vol. 4, pp. 63-72. Pergamon Press 1966. Printed in Great Britain.
19. www.faunaeur.org/distribution.php, 2011

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη καταγραφή του *Lixus umbellatarum* Fabricius (Coleoptera: Curculionidae) στην Ελλάδα. Η παρουσία του στο φαρμακευτικό φυτό *Opopanax chironium*

Κ.Θ. Μπουχέλος και Ε. Ευεργέτης

Περίληψη Το καλοκαίρι του 2004, παρατηρήθηκαν τέλεια άτομα κολεοπτέρου εντόμου της οικογένειας Curculionidae να διατρέφονται σε στελέχη φυτών *Opopanax chironium* στο Όρος Παρνασσός, σε υψόμετρο 1850 μ. Το ρητινώδες κόμμι που εκκρίνεται από τις οπές των νυγμάτων του εντόμου, είναι ο γνωστός από την αρχαιότητα, πλούσιος σε φαρμακευτικές και άλλες χρήσιμες ιδιότητες «οποπάναξ» ή «πανάκεια», ο οποίος έδωσε το όνομα του γένους στο εν λόγω φυτό. Το έντομο προσδιορίστηκε ως *Lixus umbellatarum* Fabr. (Col.: Curculionidae) και αποτελεί πρώτη αναφορά για την Ελλάδα.

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Bioaccumulation of thiram in *Mytilus galloprovincialis* and its effect on different tissues

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Summary The accumulation of thiram, a characteristic dithiocarbamate fungicide, in *Mytilus galloprovincialis* soft tissues and its effect on bivalvian DNA integrity has been examined through a series of *in vivo* exposures of mussels to 0.1, 1.0 and 10.0 mg thiram/L in saltwater for 48 h. Regarding bioaccumulation of the fungicide, a dose-dependent increase of the sodium adduct ion of thiram was observed in mussel soft tissues after the end of the exposures. This identification/quantification of thiram and one of its metabolites was achieved by Liquid Chromatography Mass Spectrometry (LC-MS) analysis. Regarding DNA integrity (DNA Single Strand Breaks) these were strongly dose-dependent. They were also tissue-dependent in concordance with the different susceptibility of certain tissues to pollutants and the levels of metabolism that takes place in them. Further investigation in oxidative and apoptotic DNA damage revealed that a) oxidative stress was evident in all the tissues examined b) apoptotic cell morphology was detected in gill and digestive gland. Imbalance of the antioxidant/prooxidant status in favour of the latter and initiation of apoptosis may be a causative mechanism of DNA damage in *M. galloprovincialis*, as it has already been shown for DNA damage caused by thiram in mammals.

Additional Keywords: apoptosis, dithiocarbamates, LC-MS, mussel, oxidative stress, Single Strand Breaks

Introduction

The dithiocarbamate class (DTCs) of fungicides comprises a group of plant protection products (PPP) which are widely used worldwide. They are the main group of fungicides used to control approximately 400 pathogens of more than 70 crops and are registered in all the EU member states and many other countries (18). They are of relatively low acute toxicity for humans with an average LD50 of more than 2523 mg/kg bw (41). Thiram (tetramethylthiuram disulfide) is one of the most characteristic dithiocarbamates. It was synthesized in 1931 and since then has been extensively used as a fungicide (41), as a seed treatment in maize, cotton and cereals (25, 35), as an accelerator and vulcanising agent during rubber processes as well as for treatment of human scabies and in antiseptic soaps and preparations (21, 22). It is also an intermediate metabolite of two other dithiocarbamates-ferbam and ziram (41). Several analytical methods are available for the detection and quantification of thiram and its metabolites. Gas chromatographic techniques have been applied with determination of carbon disulfide (CS_2), the product of the rapid degradation of DTCs. However, these methods are laborious and this has directed scientists to the exploitation of LC-MS methods which offer the advantage of the direct determination of DTCs and thiram in particular.

Thiram, like the majority of dithiocarbamates, exerts its toxic action via creation of metabolites of carbon disulfide (43) and it is of low mammalian toxicity (17). However, in certain animal models it has caused hepatotoxicity (24, 25) and adverse developmental and reproductive effects (24, 27, 40). It has also caused eczema, contact dermatitis and skin lesions to exposed workers (21, 22, 40).

Since thiram is so extensively used, it is commonly found in aquatic environments (31). According to U.S. Environmental Pro-

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tection Agency "thiram is expected to be sufficiently mobile and persistent in some cases to reach surface waters in concentrations high enough to impact aquatic life" (20). Moreover, it may not be readily catabolised and it may persist in soil for several weeks.

A series of *in vivo* experiments have shown that thiram may affect certain biological or biochemical aspects in aquatic organisms namely cladocerans (31), fish (6, 42) and mussels (15). The aim of the present project was to test for bioaccumulation and effects of thiram, which is *in vivo* non-clastogenic for mammals (19) in the model marine mussel of the Mediterranean Sea *Mytilus galloprovincialis*, in relation to tissue and dose. Following positive results, mechanistic aspects of this interaction have been sought (oxidative DNA damage and early apoptotic DNA damage). These pathways are implicated in thiram toxicity in mammals but they have not yet been examined in bivalve species.

Materials and Methods

Test animals

Commercially available *Mytilus galloprovincialis* from a mussel farm in Evia, Greece were purchased. Mussels of similar size (8-9 cm) were kept in continuously aerated glass aquaria of 15 L saltwater (salinity 33‰) at ambient temperature of 25°C, under natural light. The mussels were fed with approximately 0.05 g powdered *Spirulina* (M.Rohrer, Netherlands) every day. Nitrate and nitrite levels were periodically checked (API pharmaceuticals, USA) and they did not exceed 0.5 and 0.25 μg mL^{-1} respectively. The mussels were acclimatized in laboratory conditions for at least 5 days before the beginning of the experiments. These species absorb quickly contaminants from their environment but they depurate in an equally rapid manner when found in clean water (2).

Fungicide and Solvents

A stock solution of 10 mg thiram/L was prepared fresh in distilled water from commercially available thiram 80% w/w and was diluted to nominal concentrations of 0.1, 1.0 and 10.0 mg thiram/L in saltwater. Thiram analytical standard was purchased from Fluka (Buchs, Switzerland) and a stock solution of 100 μg mL^{-1} was prepared in methanol. The working solutions were prepared from this stock solution in methanol. Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany) and were LC-MS grade. Ethyl acetate (pro analysi) was also purchased from Merck (Darmstadt, Germany).

Experimental set up

Healthy mussels from the aquaria were exposed in 2 L glass beakers (Simax, Czech Republic) at a density of 4 mussels/beaker to final concentrations of the agrochemical stated in 2.2. The exposures lasted for 48 h after which the animals were sacrificed. The water was changed every 12 h and was spiked with thiram after each renewal. During the exposure the animals were not fed. The general condition of the animals and the mortalities were recorded daily.

Chemical analysis

Soxhlet Extraction

Lyophilized mussels (7 g) were placed with equal amount of sodium sulfate at the bottom of the Soxhlet apparatus covered with glass wool. The solvent of choice was a mixture of ethyl acetate/hexane (1:1 respectively, 200 mL) and the extraction time was 4 h. After the extraction the solvent was removed in vacuum and the mixture was reconstituted with ethyl acetate (2 mL).

Removal of sulfur compounds and Solid Phase Extraction (SPE)

The removal procedure was the following: 1 g of activated copper was added to the reconstituted mixture (2 mL) and stirred vigorously for 2 min. Centrifugation (Heraeus Labofuge 400R Thermo Electron Corporation) of the mixture was followed by the separation of the organic phase. Then the organic phase was passed from Flori-

sil SPE cartridge (Waters, SEP-PAK® Cartridges), filtered (Whatman, Puradisc™ 25 TF filters, 0.45 µm), evaporated under a gentle stream of nitrogen, reconstituted with acetonitrile (0.5 mL) and 10 µL were injected to Liquid Chromatograph Mass Spectrometer (LC/MS).

Experimental Procedure for Extraction of Thiram from Water Samples

An aliquot of 2 mL of water sample was mixed with 2 mL of ethyl acetate (vortex, a MS1 Minishaker, IKA) for 2 minutes. Then the mixture was placed in an appropriate falcon tube (15 mL) and centrifuged at 4000 rpm for 5 min, at 4°C. The organic layer was collected, filtered (Whatman, Puradisc™ 25 TF filters, 0.45 µm), evaporated under a gentle stream of nitrogen, reconstituted with acetonitrile (0.5 mL) and then an amount of 10 µL was injected for analysis to the Liquid Chromatograph Mass Spectrometer.

Liquid Chromatography Mass Spectrometry

A Shimadzu (Kyoto, Japan) LCMS-2010 EV Liquid Chromatograph Mass Spectrometer instrument was used with the LCMS solution version 3.0 software consisting of a SIL-20A prominence autosampler and an SPD-M20A diode array detector. The latter were coupled in series with a mass selective detector equipped with an atmospheric pressure ionization. The LC separation was achieved working in positive Electron Spray Ionization (ESI) mode, on a Shim-Pack XR-ODS 2.2 µm, 100×4.6 mm i.d. chromatographic column using a gradient system consisting of methanol and water. The flow rate was set at 0.8 mL min^{-1} and the column gradient program consisted of 40% methanol and 60% water, ramped linearly over the course of 7.5 min at 70 % methanol. Then methanol returned in the course of 2 min at 60% concentration and the mobile phase was held at that composition from 9.5 min to 15 min.

Validation procedure

For the validation procedure the following parameters were determined: linearity, repeatability, reproducibility, analytical Limit of Detection (LOD) and Limit of Quantification (LOQ), recoveries and matrix dependent variations as it is established by the EU guidelines. Linearity and matrix effect were assessed by analyzing standard solutions and matrix matched standards at six points in the range of 0.20-7.88 µg mL^{-1} to cover the expected range of concentrations in samples. Recovery values derived from fortified experiments at two levels of concentration and were considered as the measure of the trueness of the analytical method. For repeatability and reproducibility studies of the LC-MS methodology, five replicate determinations on the same day and on five different days of a standard solution (0.78 µg mL^{-1} of thiram) were performed. Repeatability and reproducibility is considered acceptable when relative standard deviation values (RSD%) are < 20%. Based on the statistical definition 3.3 $(S_{y/x})/a$ and $10(S_{y/x})/a$, of the LOD and LOQ respectively, they were determined at the concentration levels ranging from 0.20 to 7.88 µg mL^{-1}. $S_{y/x}$ represents the residual standard deviation and a is the slope of the respective calibration plot.

Tissue preparation for biochemical assays

The three main tissues of mussels (gill, digestive gland and haemolymph) were extracted from sacrificed animals after the end of the experiment. Briefly, for gills, the valves of the mussel were fully opened with a metallic scalpel and wet gill tissue (< 0.2 g) was extracted. The gill was gently disaggregated in HEPES buffered saline (2 mL). After centrifugation a fraction of the pellet was rediluted in saline (100 µL) and kept on ice until further processing. For haemolymph the valves of the mussel were slightly opened and haemolymph was abstracted from the posterior adductor muscle according to Rank and Jensen (36) with a 23G needle and a 1 mL syringe (PiC Insumed, Italy). Approximately 100 µL were abstracted from each animal and used without further treatment. Digestive gland suspension preparation was performed according to Birmelin *et al.* (8) with minor modifications. Briefly,

digestive glands (< 0.2 g) were abstracted from the animal and cut in small pieces with surgical scissors. The pieces were gently disaggregated in HEPES-NaOH buffered saline (10 mL) and agitated on a rocking platform (Innova2000, Brunswick Scientific) on ice for 2 h. Every 30 min aliquots of cell suspension (2 mL) were taken out and replaced with new HEPES-NaOH buffered saline. All the aliquots from the same animal were finally pooled (8 mL). After centrifugation a fraction of the pellet was rediluted in saline (100 µL) and kept on ice until further processing. The preparations provided whole and single cells with negligible additional damage.

Single Cell Gel Electrophoresis on mussel tissue (Comet assay)

A fraction of the cell suspension (15 µl) was mixed with 150 µL low melting point agarose. The mixture was spread on an agarose-precoated slide and lowered in lysis buffer (NaCl 2.5M, Na_2EDTA 0.1M, Tris base 10 mM, Triton-X 1% v/v and DMSO 10% v/v, pH 10.0) for 1 h at 4° C in the dark. Two slides per animal were prepared. The slides were then rinsed in distilled water (1mL/slide) and left in electrophoresis buffer (pH 13.0) in a horizontal electrophoresis tank (Cleaver Scientific, Ltd) for 30 min. The slides were subsequently subjected to electrophoresis at 25 V for 20 min, neutralised with Tris buffer (Tris 0.4M, pH 7.5) and stained with propidium iodide (2.5 µg/mL) (Research Organics, Cleveland, USA). Each slide was analyzed using a fluorescent microscope (Zeiss AxioCam MRC, Carl Zeiss Inc., Germany) at 200 x magnification, with an excitation filter of 515-560 nm and a barrier filter of 590 nm and scored using an image analysis package (TriTekCometScore™). 40 randomly selected nucleoids were analyzed per slide in two slides so that a total of 80 cells (per animal) were scored.

Modified Single Cell Gel Electrophoresis (Comet coupled with formamidopyrimidine glycosylase)

The procedure was as described in 2.6 with the exception that, after lysis and before unwinding in high pH buffer, the slides were rinsed 3 times in 1mL of Fpg buffer (KCl 0.1M, HEPES 40 mM, Na_2EDTA 0.5mM, bovine serum albumin 0.2 mg/mL, pH 8.0) each. Four slides per animal were prepared. One slide per pair was incubated with one unit of Fpg enzyme (AMS Biotechnology, UK) in Fpg buffer (50 µL) for 1 h as described by Collins *et al.* (14). The remaining slide of each pair was incubated with 50 µL Fpg buffer only. 80 randomly selected nucleoids were analyzed from the non-Fpg incubated slides and 80 randomly selected nucleoids were analyzed from the Fpg incubated slides. A total of 160 cells were scored. The net difference (Fpg-incubated minus non Fpg-incubated) is proportional to oxidative DNA damage.

Modified Single Cell Gel Electrophoresis (Halo assay)

The procedure was run as described in 2.6 with the complete omission of the electrophoresis step. A positive apoptosis control was co-evaluated: staurosporine (Sigma-Aldrich) was diluted in DMSO to a final concentration of 1 µM (26) and was injected (100 µL) in the adductor muscle directly below the mantle of two individuals. The animals were returned to a plastic aquarium of 1 L and sacrificed 4 h post-injection. Selected tissues were collected and processed together with the thiram samples. 50 randomly selected nucleoids were checked per slide in two slides so that a total of 100 cells per animal were scored. The percentage of characteristic halo images of apoptotic cells (39) was calculated.

Statistical Analysis

Differences between groups for SSB were assessed using the parameter %DNA in tail. Normality of data was tested by the Shapiro-Wilk *W*-test. Since data did not follow a Gaussian distribution median values were used for each set of cells (16). Data were analyzed by 2-way ANOVA. Means were separated by Tukey's HSD test (α=0.05). Differences between groups for % Fpg sensitive sites and for % apoptotic cells were assessed by Student's *t*-test. Analyses were conducted using the statistical package JMP.

Results

Validation results

The analytical method applied produced good response of linearity in the range of 0.20-7.88 μg mL^{-1} with correlation coefficient value $r^2 = 0.998$ (equation, y=241932x-4695). LOD and LOQ were determined from the calibration plot and were 0.32 μg mL^{-1} and 0.96 μg mL^{-1} respectively. Satisfactory results were obtained for all levels with recoveries for low and high concentrations, well above the cut off value of 70%. RSDs % for both low and high concentrations were < 5% therefore acceptable. RSDs % for repeatability and reproducibility were 3.69% and 4.49% respectively. Finally no matrix effect was observed.

Identification of thiram, accumulation in mussel soft tissues and concentration levels in aqueous solutions

The identification of thiram (6.61 min, retention time) was achieved by the LC-ESI-MS system functioning on the positive mode. Selected Ion Monitoring mode (SIM mode) was applied and the characteristic sodium adduct $[M+Na]^+$ ion of thiram was observed (Figure 1). Accumulation of thiram in mussel soft tissues for exposure of 0.1 mg/L (100 μg/kg) was below the LOD and thus not quantitated. Accumulation for the 1.0 and 10.0 mg/L (1000 and 10000 μg/kg correspondingly) exposure group was 375 μg/kg d.w. and 3115 μg/kg d.w respectively (Table 1). Next step was the determination of the actual concentrations of thiram in aqueous solutions. Thus three different concentration levels were investigated same with those presented in Table 1. Surprisingly for 0.1 and 1 μg mL^{-1} the only peak which appeared (3.55 min, retention time) and was identified was the peak of another sodium adduct $[C_3H_7NS_2+Na]^+$ (47). It seems that in more dilute aqueous solutions of thiram the peak of $[C_3H_7NS_2+Na]^+$ predominates. One possible fragmentation pathway is depicted in Figure 2. The aqueous solution of 10 μg mL^{-1} contained both sodium adducts $[M+Na]^+$ and $[C_3H_7NS_2+Na]^+$ as it is shown in Figure 3. For the sample of 10 μg mL^{-1}, thiram was determined as the sum of areas of peaks which correspond to thiram and its metabolite as equivalent of thiram. The sum concentration measured was 8.19 μg mL^{-1}.

Mussel mortality

The general condition of the animals (i.e. reaction to stimuli, excretion of mucus, attachment to glass-walls) was recorded daily and dead animals were discarded as soon as possible. Profuse mucus secretion was detected only in the high dose groups. Mortalities were low and did not exceed 12.5 % in all exposed groups.

SSB in relation to dose group and tissue type

SSB values were affected by both tissue and dose, while there was significant interaction between dose and tissue (Table 2). Since significant interaction was observed, multiple comparisons were conducted at each tissue for all doses and at each dose for all tissues (Figure 4).

For gill, exposure to 0.1 mg/L thiram caused a statistically significant increase in SSB (see also Figure 5A,B). Higher doses (1.0 and 10.0 mg/L) also caused an increase in relation to control but these were indistinguishable from each other. For haemolymph, exposure to the high dose only (10.00 mg/L) caused an increase in relation to control whereas the low and medium dose did not produce a statistically significant increase in SSB. For digestive gland there was also a dose response with the low dose group being indistinguishable from the control group and the medium and high dose groups being different from the control group but not different from each other.

Oxidative DNA damage in relation to dose group and tissue type

For gill, exposure to the medium dose of 1.0 mg/L caused a very significant increase ($P<0.001$) in Fpg-sensitive sites which correspond to oxidative DNA damage (see also Figure 5C). For haemolymph and digestive gland, exposure to the medium dose of 1.0

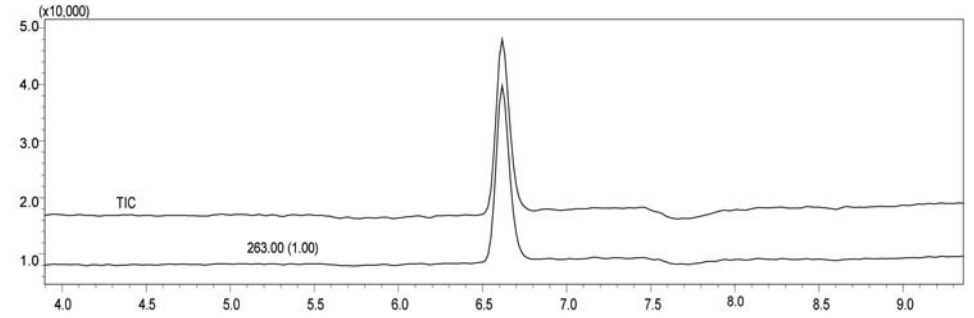

Figure 1. Total Ion Chromatogram (TIC) of Thiram in mussels sample at 0.788 μg mL^{-1} and the chromatogram of its Sodium Adduct (m/z 263).

Table 1. Accumulation of thiram in mussel soft tissues.

Treatment	Thiram (μg/kg d.b.w.)
Control	n.d.
100 μg/kg	n.d.
1000 μg/kg	375
10000 μg/kg	3115

n.d.: non detected

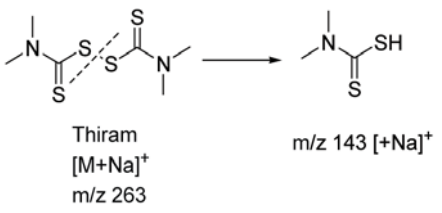

Figure 2. ESI-MS fragmentation of Thiram.

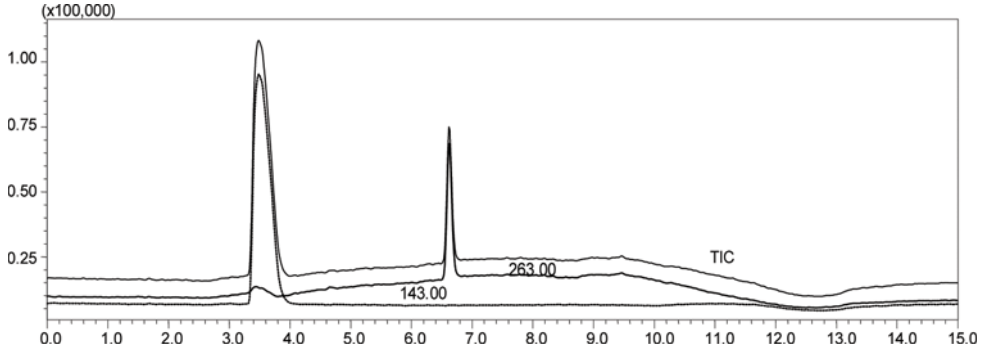

Figure 3. Total Ion Chromatogram (TIC) of Thiram in aqueous solution at 10 μg mL^{-1}. The SIM chromatograms of its Sodium Adduct (m/z 263) and the other identified adduct at m/z 143.

Table 2. F- values of the effect of tissue and dose and their interaction on SSB values for *Mytilus galloprovincialis* (2-way ANOVA).

	df	MS	F	p
Tissue	2, 24	234.02	12.32	0.0002
Dose	3, 24	993.18	52.31	<0.0001
tissue/dose	6, 24	128.82	6.78	0.0003

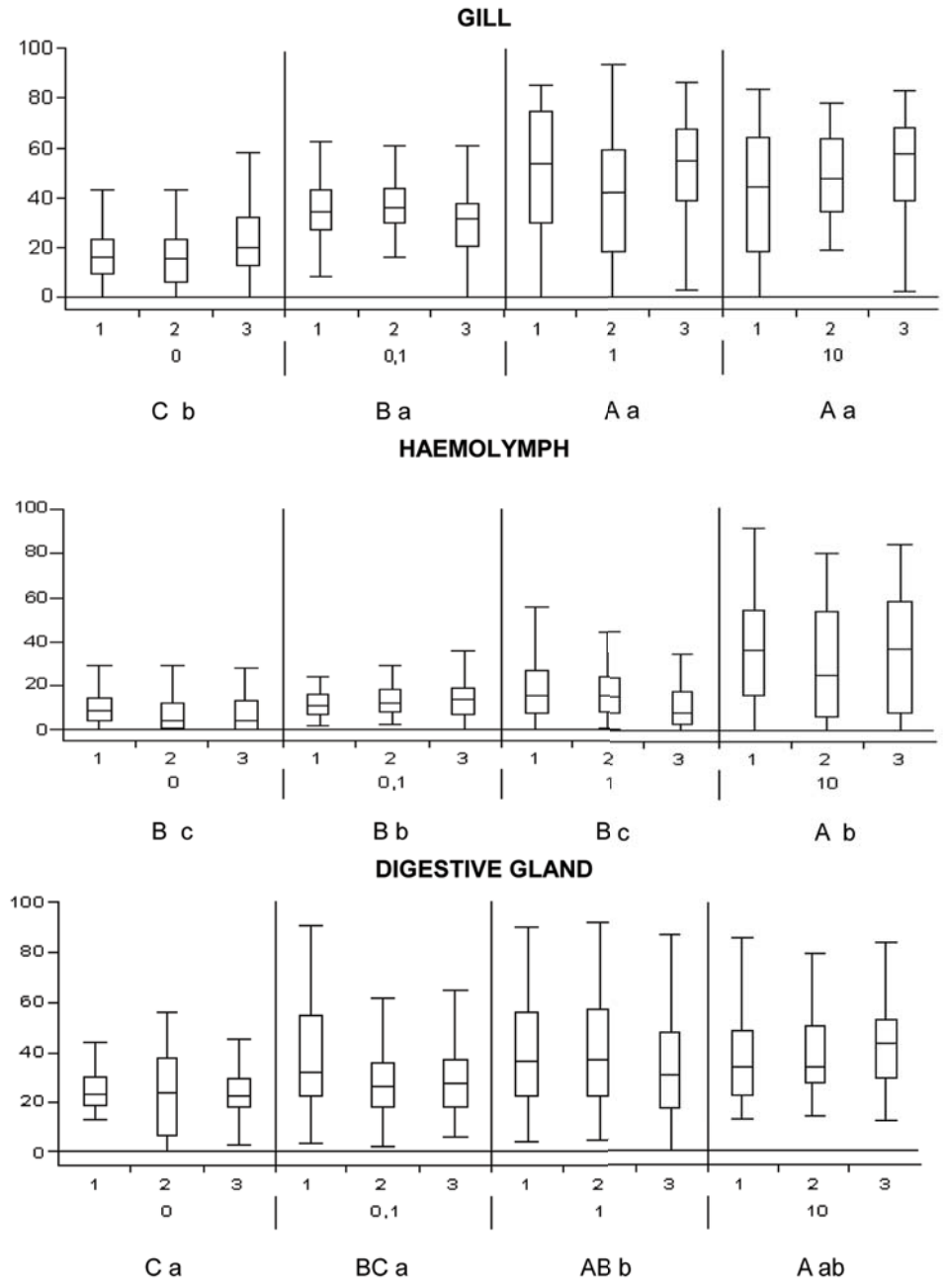

Figure 4. Percentiles of the values of SSB for each tissue of the 3 mussels in each group. Values followed by different letter differ significantly (Tukey's HSD test at α=0.05). Capital letters (A, B, C) indicate differences within tissue among concentrations while small letters (a, b, c) indicate differences among tissues within a concentration.

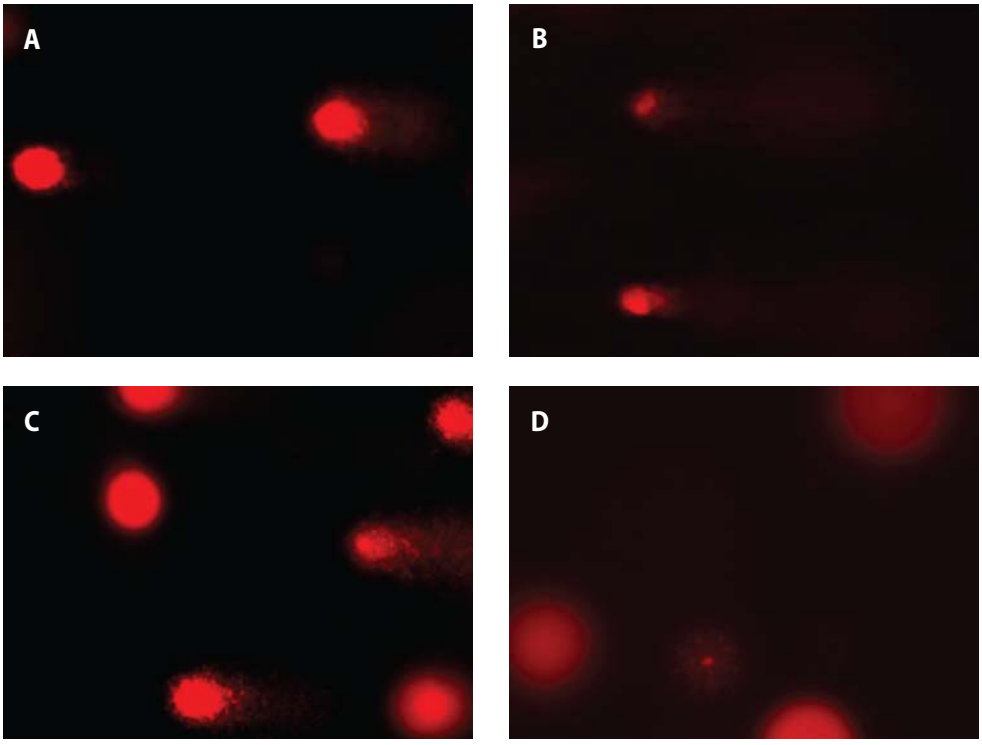

Figure 5. PI stained nuclei (A): control, gill (B): high dose, gill (C): Fpg-treated, medium dose, gill (D): apoptotic cell, medium dose, gill.

Table 3. Percentage of apoptotic cells and of Fpg-sensitive sites in mussels exposed to 1.0 mg/L thiram (*: P<0.05 in relation to control, ** P<0.01, ***P<0.001).

Treatment	Apoptotic cells (%)		
	Haemolymph	Gill	Digestive gland
Control	0	0.25 ± 0.5	0.5 ± 0.6
1.0 mg/L	0	2 ± 0.41*	1.5 ± 0.6*
	% Fpg sensitive sites		
	Haemolymph	Gill	Digestive gland
Control	0.37 ± 0.05	3.96 ± 1.91	12.46 ± 3.57
1.0 mg/L	3.80m ± 0.76**	28.04 ± 6.93***	27.79 ± 6.09**

mg/L caused a significant increase in Fpg-sensitive sites (Table 3).

Apoptotic DNA damage in relation to dose group and tissue type

For gill and digestive gland, exposure to the medium dose of 1.0 mg/L caused a significant increase in halo cells of similar morphology to the cells observed in the staurosporine-injected mussels. These cells are characteristic of apoptotic DNA damage (see also Figure 5D). For haemolymph, exposure to the medium dose of 1.0 mg/L did not cause a significant increase in halo cells. Staurosporine injection caused a significant increase in apoptotic cells (P<0.001)

for all cell types in relation to control. Injection and/or DMSO does not cause additional DNA damage as proved by a series of experiments in tissues of *Mytilus edulis* and *Mytilus galloprovincialis* (data not shown)

Discussion

Even though thiram is toxic to fish (19), bivalves (*Unio tumidus*) were able to withstand 0.1 mg/L thiram for 3 days without mortalities (15). Taking into account the apparent lack of thiram LC50 for bivalves in bibliography, the aforementioned concentration was used as the lowest one which can elicit biochemical responses. This concentration elicited responses in some of *M. galloprovincialis* tissues as mentioned later in this section.

Usually, the determination of thiram is associated with its decomposition to carbon disulfide (CS_2) in acidic medium, followed mainly by spectrometry (5, 12, 38) and head space gas chromatography (1, 34). The drawback of these methods is that they are time consuming and they also lack of selectivity or sensitivity Another way to detect DTCs is to employ Liquid Chromatography (LC) and capillary electrophoresis (CE) with UV and/or electrochemical detection. Recently Blasco *et al.* (10) applied a quantitative matrix solid-phase dispersion and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) method for the simultaneous analysis of DTCs and their degradation products in plants. Among the compounds analyzed was thiram which was detected by both APCI and ESI methods with different corresponding ions each time. This method was also applied here and was able to detect concentration-related differences in the way mussels bioaccumulate thiram.

Even though the concentrations tested were high, only certain signs of morbidity (profuse mucus secretion and delayed reaction to stimuli) were observed. Similarly, high concentrations of agrochemicals have not produced mortalities in bivalves (37).

Thiram is not considered genotoxic *in vivo*, despite being Ames positive (19). *In vitro* alkaline filter elution has also produced positive results (increase in SSB) in rat and human cells (9). The discrepancy between *in vivo* and *in vitro* results for thiram is further mirrored in the experiments conducted by Villani *et al.* (45), where exposure of mice to the maximum tolerated dose caused a borderline increase in SSB in lymphocytes and no increase in splenocytes. In contrast, concomitant exposure of human lymphocytes to thiram *in vitro* caused a significant increase in SSB. Our results for *Mytilus galloprovincialis* can therefore mostly relate to the *in vitro* outcomes of Bjørge *et al.* (9) rather than to the *in vivo* ones of Villani *et al.* (45). Bivalves possess unique physiology and biochemistry quite different from higher organisms. As a result, innocuous substances for higher organisms may be biologically active for bivalves and vice versa.

The effect of thiram (SSB increase) was different in each tissue as shown in Figures 4 and 5 and there was a significant interaction between dose and tissue (Table 2). The route of exposure (water-spiking) may be an important factor for gill susceptibility since gill cells would be the first ones to come into contact with thiram. The direct contact of the large surface area of gills with the diluted pollutants may also contribute to this (3). This difference may also stem from the cell subpopulations: whereas the main gill cell type and the agranular haemocytes of *Mytilus galloprovincialis* were sensitive to the genotoxic effects of benzo[a]pyrene (BaP), the agranular ones which are present at varied concentrations were relatively refractory to these effects (44). Regarding the digestive gland, it is considered to be the main organ of metabolism of organic xenobiotics and the main site of biotransformation enzyme activities (8). As a result a number of reactive intermediates produced may directly attack vicinal digestive gland DNA. Furthermore, the highly condensed chromatin of digestive gland nuclei which creates additional alkali labile sites also contributes to % tail DNA (33). This higher baseline damage may have masked here the effect of thiram, at least in

the low dose group.

Thiram, in the present experiments, has caused a significant increase in oxidative DNA damage as measured through Fpg incubation, which quantifies mainly 8-oxo-dG but also other damaged purines according to Collins *et al.* (14). This increase was most prominent in gill cells rather than in haemocytes and in digestive gland cells. The metabolic system of bivalves, even though markedly different from the mammalian ones is capable of producing oxidizing intermediates (28) which in their turn affect the cell's metabolism and components (46). For example, in a similar way to the present results, the common aquatic pollutant BaP which necessitates metabolic activation to redox quinones to exert its toxic action, caused formation of 8-oxo-dG in *Mytilus galloprovincialis* digestive gland (2, 29).

An oxidative mode of action is not unusual for thiram as it has been shown in other animal models. Any imbalance between prooxidant substances and antioxidant defenses in favor of the prooxidants causes oxidative stress associated with damage to cellular macromolecules (40). Thiram has acted as a potent oxidative agent in the liver of broilers fed with this fungicide. The activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were decreased and this lowering of antioxidant defenses has led to lipid peroxidation in the liver of broilers (27). Thiram has also caused deregulation in activities of key antioxidant enzymes (catalase, GSH-Px, SOD, glutathione reductase) in an *in vitro* model of V79 Chinese hamster ovary cells (22). These results may be partly explained by glutathione (GSH) depletion caused by thiram (13, 23). GSH offers one of the most efficient non-enzymatic protective mechanisms by its conjugation with electrophilic and/or oxidised components. Thiram however possesses a reactive disulfide bond which may react with thiols of critical cellular proteins such as GSH forming mixed disulfides and other products (23), thus rendering them inactive. Furthermore, the GSH redox cycle offers the reducing equivalents for thiram reduction in the cell, minimizing in this way the regeneration of reduced GSH. In more detail, it is postulated that dithiocarbamates undergo oxidation by Cu^{2+} ions within the cell to their corresponding thiuram disulfides. These intermediates are then reduced by GSH, regenerating the parent compound and oxidized glutathione (11). GSH aberrant metabolism due to thiram was further corroborated in studies in V79 CHC which showed a decrease in total GSH/oxidised GSH ratio (22). Therefore, the pro-oxidant effects of thiram are considered to be indirect and mainly due to the lowering of antioxidant defenses.

Finally, a series of important experiments in aquatic organisms showed that thiram oxidative effects are not peculiar to terrestrial organisms. Namely, incubation of *Oncorhynchus mykiss* liver with thiram led to loss or decrease of activity of SOD and GSH-Px respectively (6). Exposure of the mussel *Unio tumidus* to thiram has also caused decrease of activity of selenium-dependent glutathione peroxidase and glutathione reductase as well as decrease in reduced and oxidised GSH in both gills and digestive gland (15).

Regarding testing for apoptosis, apoptotic cells give a characteristic image of large fan-like tail and small head (ghost cells) in the conventional comet assay. However due to their extensive fragmentation, they may become lost during the electrophoresis step (32). In contrast, omission of the electrophoresis step but retention of alkaline unwinding in the alkaline halo assay depicts successfully the unique morphology of apoptotic cells which present a difuse, spotted halo and a pin-like head clearly delineated from the halo (39). The assay has here revealed a significant increase in apoptotic cells in gills and digestive glands but no increase in haemolymph when mussels were exposed to 1.00 mg/L thiram (approximately 4 μM) for 48 h. In related bibliography, triclosan (3 nM) caused more extensive damage (approximately 16%) after 48 h exposure to *Dreissena polymorpha* haemolymph (45). This difference cannot be readily explained, however it may be linked to the apoptotic

potency of the tested chemical, the mussel species used and possibly the lower sensitivity in our experiments (staurosporine positive control also caused less than 16% apoptotic cells). An apoptotic mode of action, as this recorded here for *Mytilus galloprovincialis* gill and digestive glands, has been shown in a variety of animal models for thiram. Disarrangement of redox related mechanisms and disintegration of important thiol-containing proteins eventually led to apoptotic cell death in the V79 CHC cells (22). Thiram has also caused apoptosis in avian lymphocytes (4), in bovine capillary endothelial cells (30) and in PC12 cells, the latter due to massive Ca^{2+} intracellular influx (24).

Overall, a simple and convenient analytical method for the determination of the concentration of thiram in mussel samples revealed that *M. galloprovincialis* may accumulate this fungicide in a dose-dependent way. This accumulation elicited dose- and tissue-related SSB and it also caused increased 8-oxo-dG levels (in all tissues) and % apoptotic cells (in gill and digestive gland). The two last parameters have also increased in a number of *in vivo* mammalian models. It is possible that a common mechanism (reduction, depletion or disintegration of important thiol-containing proteins) is implicated in both mammalian and non-mammalian thiram toxicity.

Literature cited

1. Ahmad, N., Guo, L., Mandrakas, P. and Appleby, S. 1995. Determination of dithiocarbamate and its breakdown product ethylenethiourea in fruits and vegetables *J. AOAC Int*., 78: 1238-1243.
2. Akcha, F., Izuel., C., Venier, P., Budzinski, H., Burgeot, T. and Narbonne, J.-F. 2000. Enzymatic biomarker measurement and study of DNA adduct formation in benzo[a]pyrene-contaminated mussels, *Mytilus galloprovincialis*. *Aquatic Toxicology*., 49: 269-287.
3. Almeida, E.A, Bainy, A.C.D., Dafre, A.L., Gomes, O.F., Medeiros, M.H.G. and Di Mascio, P. 2005. Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed. *Journal of Experimental Marine Biology and Ecology*, 318: 21– 30.
4. Ambwani, S., Ambwani, T.J. and Chauhan, R.S. 2008. Evaluation of thiram induced oxidative stress, immunosuppression and apoptosis in avian lymphocytes culture system. *Journal of Biotechnology*, 136: S620-S632.
5. Association of Official Analytical Chemists, 2000. Official Methods of Analysis, 16th ed., AOAC, Arlington, USA
6. Binelli, A., Cogni, D., Parolini, M., Riva, C. and Provini, A. 2009. In vivo experiments for the evaluation of genotoxic and cytotoxic effects of Triclosan in Zebra mussels hemocytes. *Aquatic Toxicology*. 91: 238-244.
7. Babo, S. and Vasseur, P. 1992. *In vitro* effects of Thiram on liver antioxidant enzyme activities of rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, 22 : 61-68.
8. Birmelin, C., Mitchelmore, C.L., Goldfarb, P.S. and Livingstone, D.R. 1998. Characterisation of biotransformation enzyme activities and DNA integrity in isolated cells of the digestive gland of the common mussel, *Mytilus edulis* L. *Comparative Biochemistry and Physiology Part A*, 120: 51-56.
9. Bjørge, C., Brunborg, G., Wiger, R., Holme, J.A., Scholz, T., Dybing, E. and Søderlung, E.J. 1996. A comparative study of chemically induced DNA damage in isolated human and rat testicular cells. *Reproductive Toxicology*, 10: 509-519.
10. Blasco, C., Font, G. and Picó, Y. 2004. Determination of dithiocarbamates and metabolites in plants by liquid chromatography-mass spectrometry. *Journal of Chromatography A*, 1028: 267-276.
11. Burkitt M.J., Bishop, H.S., Milne, L., Tsang S.Y., Provan G.J., Nobel C.S.I., Orrenius S. and Slater, A.F.G, 1998. Dithiocarbamate toxicity towards thymocytes involves their copper-catalyzed conversion to thiuram disulfides, which oxidize glutathione in a redox cycle without the release of reactive oxygen species. *Archives of Biochemistry and Biophysics*, 353: 73-84.
12. Caldas, E.D., Conceicao, M.H., Miranda, M.C.C., de Souza, L.C.K.R. and Lima, J.F. 2001. Determination of Dithiocarbamate Fungicide Residues in Food by a Spectrophotometric Method Using a Vertical Disulfide Reaction System. *Journal of Agricultural and Food Chemistry*, 49: 4521-4525.
13. Careser, C., Boget, S., Parvaz, P. and Revol, A. 2001. An evaluation of thiram toxicity on cultured human skin fibroblasts. *Toxicology*, 162: 89-101.
14. Collins, A.R., Dobson, V.L., Dusinska, M., Kennedy, G. and Stetina, R. 1997. The comet assay: what can it really tell us? *Mutation Research*, 375: 183-193.
15. Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M. and Vasseur, P. 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the

gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquatic Toxicology*, 39: 93-110.

16. Duez, P., Dehon, G., Kumps, A. and Dubois, J. 2003. Statistics of the Comet assay: a key to discriminate between genotoxic effects. *Mutagenesis*, 18: 159-166.
17. Edwards, I.R., Ferry, D.H. and Temple, W.A. 1991. Fungicides and related compounds. In: Hayes, W.J. *et al.* (Eds.), Handbook of Pesticides Toxicology. Academic Press, New York, pp. 1436-1469.
18. European Commission, Food Safety, Pesticide Residues, Brussels, 2002, http://europa.eu.int/comm/food/fs/ph_ps/pest/index_en.htm
19. European Commission- Health & Consumer Protection Directorate. 2003. Thiram, 6507/VI/99-Final.
20. Filipe, O.M.S., Vidal M.M., Duarte, A.C. and Santos E.B.H. 2008. Influence of fulvic acids and copper ions on thiram determination in water. *Journal of Agricultural and Food Chemistry*, 56: 7347-7354.
21. Girotti, S., Maiolini, E., Ghini, S., Ferri, E., Fini, F., Nodet, P. and Eremin, S. 2008. Quantification of thiram in honeybees: development of a chemiluminescent ELISA. *Analytical Letters*, 41: 46-55.
22. Grosicka-Maciąg, E., Sadurska, B., Szumilo, M., Grzela, T., Lazarcyk, P., Niderla-Bielinska, J. and Rahden-Staron, I. 2005. Effect of glutathione depletion on apoptosis induced by thiram in Chinese hamster fibroblasts. *International Immunopharmacology*, 5: 1945-1956.
23. Grosicka-Maciąg, E., Kurpios, D., Czeczot, H., Szumilo, M., Skrzycki, M., Suchocki, P. and Rahden-Staron, I. 2008. Changes in antioxidant defense systems induced by thiram in V79 Chinese hamster fibroblasts. *Toxicology In Vitro*, 22: 28-35.
24. Han, M.S., Shin, K.-J., Kim, Y.-H., Kim, S.-H., Lee, T., Kim E., Ryu, S.H. and Suh, P.-G., 2003. Thiram and ziram stimulate non-selective cation channel and induce apoptosis in PC12 cells. *Neurotoxicology,* 24: 425-434.
25. Kumar Sharma, V., Aulakh, J.S. and Kumar Malik, A. 2003. Thiram: degradation, applications and analytical methods. *Journal of Environmental Monitoring*, 5: 717-723.
26. Kurelec, B. 1995. Inhibition of multixenobiotic resistance mechanism in aquatic organisms: ecotoxic consequences. *Science of the Total Environment*, 171: 197-204.
27. Li, J., Bi, D., Pan, S. and Zhang, Y. 2007. Effect of diet with thiram on liver antioxidant capacity and tibial dyschondroplasia in broilers. *British Poultry Science*, 48: 724-728.
28. Livingstone, D.R. 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin*, 42: 656-666.
29. Machella, N., Regoli, F. and Santella R.M. 2005. Immunofluorescent detection of 8-oxo-dG and PAH bulky adducts in fish liver and mussel digestive gland. *Aquatic Toxicology*, 71: 335-343.
30. Marikovsky, M. 2002. Thiram inhibits angiogenesis and slows the development of experimental tumors in mice. *British Journal of Cancer*, 86: 779-787.
31. Martins, J., Soares, M.L., Saker, M.L., OlivaTeles, L. and Vasconcelos, V.M. 2007. Phototactic behavior in *Daphnia magna* Straus as an indicator of toxicants in the aquatic environment. *Ecotoxicology and Environmental Safety*, 67: 417-422.
32. Meintieres, S., Nesslany, F., Pallardy, M. and Marzin, D. 2003. Detection of ghost cells in the standard alkaline comet assay is not a good measure of apoptosis. Environmental and *Molecular Mutagenesis*, 41: 260-269.
33. Mitchelmore, C.L., Birmelin, C., Livingstone, D.R. and Chipman, J.K. 1998. Detection of DNA strand breaks in isolated mussel (*Mytilus edulis* L.) digestive gland cells using the ''comet'' assay. *Ecotoxicology and Environmental Safety*, 41: 51-58.
34. Perz, R.C., Van Lishaut, H. and Schwack, W. 2000. CS2 Blinds in brassica crops: False positive results in the dithiocarbamate residue analysis by the acid digestion method. *Journal of Agricultural and Food Chemistry,* 48: 792–796.
35. Pesticide Manual, 9th Edition, 1991. In: Worthing C.R. & Hance R.J. (eds), British Crop Protection Council, Surrey, UK, pp. 822-823.
36. Rank, J. and Jensen, K. 2003. Comet assay on gill cells and hemocytes from the blue mussel *Mytilus edulis*. *Ecotoxicology and Environmental Safety*, 54: 323-329.
37. Revankar, P.R and Shyama, S.K. 2009. Genotoxic effects of monocrotophos, an organophosphorous pesticide, on an estuarine bivalve, *Meretrix ovum*. *Food and Chemical Toxicology*, 47: 1618–1623.
38. Schwack, W. and Nyanzi, S. 1994. Analysis of dithiocarbamate fungicides. Second-derivative UV-spectroscopic determination of CS_2, COS, and thiram. *Zeitschrift für Lebensmittel-Untersuchung und –Forschung*, 198: 3-7.
39. Sestili, P., Martinelli, C. and Stocchi, V. 2006. The fast halo assay: An improved method to quantify genomic DNA strand breakage at the single-cell level. *Mutation Research*, 607: 205-214.
40. Singh, B., Sharma, D.K. and Gupta, A. 2009. A study towards release dynamics of thiram fungicide from starch-alginate beads to control environmental and health hazards. *Journal of Hazardous Materials*, 161: 208-216.
41. Stevens, J.T. and Sumner, D.D. 1991. Herbicides. In: Wayland J., Hayes Jr, Edward R., Laws Jr. (eds.), Handbook of Pesticide Toxicology. Academic Press, San Diego, USA, pp. 1333-1336.

42. Teraoka, H., Urakawa, S., Nanba, S., Nagai, Y., Dong, W., Imagawa, T., Tanguay, R.L., Svoboda, K., Handley-Goldstone H.M., Stegeman, J.J. and Hiraga, T. 2006. Muscular contractions in the zebrafish embryo are necessary to reveal thiuram-induced notochord distortions. *Toxicology and Applied Pharmacology*, 212: 24-34.

43. Thiruchelvam, M. 2005. Thiram. In: Wexler P. (ed.), Encyclopedia of Toxicology, Elsevier, pp.179-181.

44. Venier, P., Maron, S. and Canova, S. 1997. Detection of micronuclei in gill cells and haemocytes of mussels exposed to benzo[a]pyrene. *Mutation Research*, 390, 33–44.

45. Villani, P., Andreoli, C., Crebelli, R., Pacchierotti, F., Zijno, A. and Carere, A. 1998. Analysis of micronuclei and DNA single-strand breaks in mouse splenocytes and peripheral lymphocytes after oral administration of tetramethylthiuram disulfide (thiram). *Food and Chemical Toxicology*, 36: 155-164.

46. Vlahogianni, T., Dassenakis, M., Scoullos, M.J., and Valavanidis, A. 2007. Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. *Marine Pollution Bulletin*, 54 :1361-1371.

47. Walia, S., Kumar Sharma, R. and Parmar, B.S. 2009. Isolation and Simultaneous LC Analysis of Thiram and Its Less Toxic Transformation Product in DS Formulation. *Bulletin of Environmental Contamination and Toxicology*, 83: 363-368.

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Μελέτη βιοσυσσώρευσης του thiram στους μαλακούς ιστούς του μυδιού *Mytilus galloprovincialis*

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Περίληψη Η βιοσυσσώρευση του thiram, ενός χαρακτηριστικού μυκητοκτόνου της κατηγορίας των διθειοκαρβαμιδικών, εξετάσθηκε όσον αφορά στους μαλακούς ιστούς του μυδιού *Mytilus galloprovincialis*. Επίσης εξετάσθηκε η επίδραση του thiram στην ακεραιότητα του DNA του *Mytilus galloprovincialis*, μέσω μια σειράς *in vivo* εκθέσεων σε 0,1, 1,0 and 10,0 mg thiram/L σε θαλάσσιο νερό για 48 h. Παρατηρήθηκε μια δοσοεξαρτώμενη βιοσυσσώρευση συμπλόκων ιόντων του νατρίου με το thiram μετά το πέρας των εκθέσεων. Αυτή η ταυτοποίηση/ποσοτικοποίηση του thiram και των μεταβολιτών του πραγματοποιήθηκε μέσω Υγρής Χρωματογραφίας Φασματομετρίας Μάζας (LC-MS). Όσον αφορά στην ακεραιότητα του DNA του *M. galloprovincialis* (παρατηρούμενες μονές αλυσίδες DNA) η εμφάνισή τους ήταν ισχυρά δοσοεξαρτώμενη. Αυτό το αποτέλεσμα επηρεάστηκε εξίσου σημαντικά από τον ιστό υπό εξέταση , όπως ήταν αναμενόμενο, δεδομένης της διαφορετικής ευαισθησίας του κάθε ιστού στο thiram και του μεταβολισμού που λαμβάνει χώρα σε αυτόν. Περαιτέρω αναλύσεις DNA βλαβών λόγω οξειδωτικού στρες και απόπτωσης κατέδειξαν πως α) οξειδωτικό στρες ήταν εμφανές σε όλους τους εξεταζόμενους ιστούς και β) κυτταρικά μορφολογικά σχήματα χαρακτηριστικά αποπτωτικών διεργασιών παρουσιάστηκαν τόσο στα βράγχια όσο και στον πεπτικό αδένα του οργανισμού. Από τα παραπάνω αποτελέσματα μπορούμε να υποθέσουμε πως ανισορροπία μεταξύ των αντιοξειδωτικών/οξειδωτικών μηχανισμών των κυττάρων του *M. galloprovincialis*, προς όφελος των δεύτερων, καθώς και έναρξη αποπτωτικών κυτταρικών διεργασιών είναι δυνατόν να αποτελέσουν τον κύριο λόγο βλαβών DNA στον *M. galloprovincialis* λόγω του thiram. Αντίστοιχοι μηχανισμοί έχει αποδειχθεί ότι ισχύουν για τα θηλαστικά.

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