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E CONSERVAÇÃO
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"In Honorium"
do Professor Catedrático Emérito

Ilídio Rosário
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Este trabalho, em honra do Prof. Ilídio Rosário dos Santos Moreira, é, também, uma homenagem à Prof. Maria Edite Texugo de Sousa, que colaborou com a autora na realização da investigação apresentada.

BIBLIOGRAFIA

AQUATIC WEED BIOLOGICAL CONTROL: OLFACTORY ATTRACTION OF Weevils Neochetina bruchi and N. eichhorniae FOR WATER HYACINTH (Eichhornia crassipes). A CASE STUDY
Luta biológica contra insetentes aquáticas: a atração olfativa dos gorgulhos Neochetina bruchi e N. eichhorniae por jacinho-aquático (Eichhornia crassipes). Um estudo de caso
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ABSTRACT
Water hyacinth, Eichhornia crassipes (C. Martius) Solms-Laubach, is one of the worst aquatic weeds in many parts of the world. Throughout this naturalized range the most significant biological control agents are two weevil species Neochetina bruchi Hustache and N. eichhorniae Warner. The use of mass trapping schemes with host-plant volatiles in order to increase the number of Neochetina spp. on areas with water hyacinth infestations has been under theoretical analysis. In an attempt to investigate the functional basis of water hyacinth selection by adults of Neochetina weevils, the studies of antennal sensory structures and the identification of the host-plant volatiles that mediate the Eichhornia-Neochetina attraction were accomplished. Antennal sensilla typography, number and placement analyzed by SEM revealed an increase in sensilla morphological diversity and number from the pedicel to the club in both species and sexes, but in small number when compared with other Coleoptera. Biosays with a y-tube olfactometer indicated that the volatiles produced by broken leaves and stems of water hyacinth were attractive to both species and sexes of weevils. The collection and analysis of these volatiles by GC-MS indicated the presence of three compounds, (Z)-3-hepten-1-ol, (Z)-3-hepten-1-ol and 2-ethyl-1-hexanol. Olfactometer results indicated that N. eichhorniae and N. bruchi males and females were attracted to (Z)-3-hepten-1-ol when tested in a y-tube but they did not respond to the other constituents. These results indicate that Neochetina weevils are responsive to water hyacinth volatiles, yet may suggest the importance of other clues (e.g. visual) rather than only olfactory in host selection, which should be further investigate to optimize the water hyacinth integrated control programs.

Key Words: Biological control of freshwater weeds, Eichhornia crassipes, Neochetina, antennal sensilla, semiochemicals.

RESUMO
A planta de jacinho-aquático, Eichhornia crassipes (C. Martius) Solms-Laubach, é considerada uma das piores insetentes aquáticas a nível mundial. Na sua área de distribuição, os agentes de luta...
biológica mais significativos são duas espécies de gorgulhos Neocentria bruchi Hustache e N. eichhorniae Warner. A utilização de voláteis da planta hospedeira em programas de armadilha para captura em massa destes insetos e posterior largada em zonas densamente infestadas de jactino-aquático tem sido alvo de discussões teóricas. Para clarificar as bases funcionais da seleção de jactino-aquático por adultos de Neocentria, procedeu-se ao estudo das estruturas sensoriais das antenas dos gorgulhos e à identificação dos voláteis da planta hospedeira, que mediam a atração *Eichhornia* -Neocentria. A tipologia, número e distribuição das sensiias antenas analisadas por MEV revelaram um aumento crescente da diversidade morfológica e do número de sensis nas regiões do pedicelo para a base da antena, em ambas as espécies e sexos, mas em número reduzido, quando comparado com outros Coleoptera. Os bioensaios realizados com oeferruto num tubo em *E* indicaram que os compostos produzidos por folhas e caules secionados de jactino-aquático foram atraentes para ambos os sexos e espécies de gorgulhos. A recolha e análise destes voláteis revelaram a presença de três compostos, (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol e 2-ethyl-hexan-1-ol. Os resultados do olfactometer indicaram que tanto machos como fêmeas de *N. eichhorniae* e *N. bruchi*, quando testados num tubo em *Y*, são atraiados para (Z)-3-hexen-1-ol, mas não respondem aos outros constituintes. Estes resultados indicam que os gorgulhos *Neocentria* spp. são sensíveis a voláteis de jactino-aquático, mas parecem indicar a importância de outros tipo de estímulos (por ex. visuais), e não apenas olfativos, na seleção da planta hospedeira, o que deve ser investigado para optimizar os programas de gestão integrada de jactino-aquático.


**INTRODUCTION:**

Water hyacinth, *Eichhornia crassipes* (C. Martius) Solms-Laubach, a Pontederiaceae, is ranked as one of the world's worst invasive water weeds causing widespread problems to millions of users of water bodies and water resources (Pentland & Earle 1948; Copal 1987). The thick mats of water hyacinth were originally seen mainly as a practical problem for fisheries and navigation, hydroelectric power generation and irrigation schemes. However, water hyacinth is also a major threat to biodiversity, affecting fish and aquatic fauna, plant community structure and diversity, and human health and water supplies (Hill et al. 2011).

It is native to the Neotropics and has spread to almost all countries with a suitable climate (Stark & Goyder 1983), and has been introduced around the world as an ornamental plant because of its attractive flowers. Extensive infestations developed in the southern USA (especially Louisiana and Florida), Mexico, Panama, many parts of Africa (especially the Nile and Congo river systems), the Indian sub-continent, South-East Asia, Indonesia and Australia (Holm et al. 1977; Copal 1987; Center 1994; Julien et al. 1999). Though water hyacinth is cultivated as an ornamental almost all over Europe, it has naturalized only in Portugal (the highest northern latitude of its ecological range) (Pieterse & Murphy 1990).

Water hyacinth was first noticed in Portugal in 1939 in Tagus basin, becoming weed in many lowland aquatic systems (Moreira et al. 1989). When compared with other countries, the problems caused by this weed are reduced in Portugal, as infestations are mostly in irrigation and drainage channels on the so-called "Lença Grande de Vila Franca de Xira", a fertile alluvial plain of the Tagus and Sorraia rivers. Nevertheless, its importance has been increased, mainly on small wetlands areas that are important biodiversity reserves and particularly valued for their bird populations (e.g. Alvare da Golegã, Lagos de Santa Margarida, Paul de Boquilobo, Paul de Madriz, and Paul da Tornada) (Moreira et al. 1989; 1999a).

The maintenance of water hyacinth populations at their lowest "feasible levels" together with the reduction in herbicide use in waterways, due to concerns for the quality of domestic and recreational use water supplies, implies the need for more biocidal forms of aquatic weed control. The possible integrated aquatic weed control approaches include: control of nutrient levels, use of booms to control movement of the weed, exploitation of variable water levels, manual removal of the weed from shores and small channels, mechanical removal or destruction by land-based or floating equipment, and use of biological control agents (Center 1994; Julien et al. 1999; Moreira et al. 1999a).

The first suggestion of using insects to control weeds was made by Assa Fitch in 1855, who suggested the importation from Europe to the USA of insects that fed on toadflax (*Linaria vulgaris*) on the old continent but not in the new one. The first actual use of insects for weed control occurred in 1863 in India when a cockroach insect (*Dactylonyx ceylonicus*) was moved from north to south to control the cactus *Opuntia vulgaris* (Van Driese & Bellows, 1996). Biological weed control has been achieved through two routes: introduction of natural enemies against adventive and native weeds (usually using agents collected from an adventive weed native range), and augmentation of natural enemies, which are released or applied at specific locations where control is needed (Walker et al. 1989).

While biological control of invertebrates (by predation and parasitism) causes direct mortality of the individuals attacked, biological control of weeds can be achieved by a variety of mechanisms, which does not necessarily include directly caused mortality of the target plant. Plants that are prevented from successfully reproducing (by flowerfeeders which destroy flowers before they can set seeds, or by seed feeders which destroy the seeds themselves) and then die naturally have been as effectively eliminated as those that are killed outright by herbivore attack (Van Driese & Bellows 1996).

Nine arthropods and three fungi have been developed and released for biocontrol of *E. crassipes* in more than 40 countries (Guido & Perkins 1975; Harley 1990; Julien & Griffiths 1998; Julien et al. 1999; Sosa et al. 2007). The arthropods are the weevils *Neocentria bruchi Hustache and Neocentina eichhorniae* Warner (Coleoptera: Elateridae); the moths *Niphograpta albignotata* (Warren) (Lepidoptera: Pyralidae), *Xylosis inselbii* (Walker) (Lepidoptera: Pyralidae), and *Bellura densa* Walker (Lepidoptera: Noctuidae); the water hyacinth bug *Ectocissus catanescens* (Carvalho) (Hemiptera: Miridae); the grasshopper *Conopia aquaticum* (Bruner) (Orthoptera: Acrididae); the leafhopper *Megalopygus sculptilis* Berg (Hemiptera: Delphacidae) and the leaf-mining mite *Orthogamasus ferox* (Wallwork) (Acari: Gamidiae). The fungi are all Hypoxylonetes: *Acronomus zonatum* (Sawada) W. Gams, *Cercospora paniphi Tharp and Cercospora rosmdni Conway*.

The two Neocentina weevils have been released into more than 30 countries and are considered the most successful projects for biological control of weeds in the world (Crawley 1989; Julien et al. 1999). They have together given significant results in Argentina, Australia, Benin, India, Mexico, Papua New Guinea, South Africa, Sudan, Tanzania, Thailand, Uganda, USA, and Zimbabwe, acting apparently in a complementary fashion (DeLuchi & Cordo 1983; Center 1994; Hill & Cilliers 1999; Julien et al. 1999). In many other countries where releases have been made, their effectiveness
has been either limited or not yet evaluated. In Portugal, for example, *Neocthena* spp. weevils, imported from Florida in 1955, were preliminary tested in quarantine but did not survive to winter (Moreira et al. 1999b). Due to EU restrictions to the use of biological control agents, native from other continents, *Neocthena* weevils were never released in the field to control water hyacinth infestations.

Adult weevils feed on the leaf and petiole surfaces, making distinctive, almost square, feeding scars. This may cause significant loss of functional leaf surface and also may allow entry of pathogens. The most significant damage, however, is caused by the larval stages which develop from eggs laid in the petiole and feed for many weeks inside the petiole tissue, migrating as necessary to new petioles as the tissue dies. This damage to the petiole often results in complete collapse of the leaf and eventually in loss of buoyancy so that the whole plant sinks.

The impact of *Neocthena* spp. in the abundance of water hyacinth has been mitigated by the current weed management practices, which rely heavily on herbicidal control (Center & Durden 1986). Moreover, the lack of effectiveness may be partially attributable to a microsporidiosis that infects Florida populations, which had provided a source of weevils for several countries (Rebelo & Center 2001), before microsporidia-free colonies have been released.

Center et al. (2002) suggest that augmentative releases of *Neocthena* weevils quickly suppress regrowth of incipient water hyacinth populations compared with natural infestations. So, the use of mass trapping schemes with host-plant volatiles in order to increase the number of biological control agents on more infested areas has been under theoretical analyses. However, an important factor compromising this augmentative control program is the lack of methods that allow for the large-scale collector or production of sufficient numbers of weevils.

Plant chemistry is likely to affect acceptability (to adults) and suitability (as food). Species of herbivores with narrow host ranges are frequently adapted to respond to specific chemicals found in their host plants. Species with broad host ranges often respond more to nonspecific stimuli coupled with the absence of specific deterrent compounds (Rausher 1992). Thus, chemical communication is emerging as an important component in IMA. Typically, attractants such as pheromones or host-produced volatiles are used to attract pestiferous insects. However, semiochemicals could be used for beneficial insects to monitor the success of biological control releases or for conserving weevils that would be destroyed by herbicide treatments. Once augmentation efforts with semiochemical attractants are feasible and implemented, the biocontrol of this weed should improve dramatically. This would significantly reduce the need for expensive and environmentally damaging weed management practices such as the direct application of herbicides into domestic water supplies.

There is evidence that semiochemicals function to concentrate *Neocthena* eichhorniae around fresh weevil-feeding damage water hyacinth leaves (De Fossé & Perkins 1977). The weevils are endemic to the Amazon basin and during the wet season and the associated flooding their host plant is patchily distributed over great distances (DeLoach 1975). Under these conditions, host volatile attraction would be highly selective. Host range tests have confirmed that these weevils are highly host specific within the plant family Pontederiacese and prefer and complete development only on water hyacinth (DeLoach 1975; Gopal 1987; Julien et al. 1999).


Although all this considerable work has been done on evaluating weevil and host-plant-produced attractants of other weevil-host plant species complexes, and despite the clear and long-standing agricultural pest status of *Eichhornia* crassipes and its biological control agents, the weevils *N. brunchi* and *N. eichhorniae* is little known about the physiological basis of the sensory aspects of host-plant recognition. While research is underway searching for an aggregation pheromone, host plant volatiles were examined as an additional component of an effective weevil trapping system (Perez et al. 1997). However, the chemical nature of this activity has never been elucidated.

Identification of water hyacinth chemicals can then be used to understand the behavior and chemical ecology of *N. brunchi* and *N. eichhorniae* and use as a mass trapping scheme to capture large numbers of live weevils for augmentative biological control of the weed. Biological control practitioners attempting to manage these beneficial weevil populations would benefit from trapping methods that monitor populations for abundance, dispersal rates, and seasonality.

How these insects locate and recognize water hyacinth plants and mate presumably depends, at least in part, on mechanical, olfactory, and gustatory sensory receptors. The efficiency of IPM strategies to control *E. crassipes* is dependent on the biological knowledge that can be achieved about the plant-weevil relationship. As the placement of olfactory sensilla on the antennae seems to be a morphological adaptation having an influence on the efficiency or sensitivity odor perception, the specific distribution pattern of olfactory receptors is related to the specific searching behavior of a species (Zacharuk 1985; Bernays & Chapman 1994). Thus, antennal olfactory sensor description and selective responsiveness of the antennal olfactory system to host-plant volatiles have been the subject of intensive scrutiny (Sass 1978; Alm & Hall 1986; Isírdor & Solina 1992; Bowen 1995; Meriée et al. 1997; Pophol 1997; Meriée et al. 1998; Barlet et al. 1999; Meriée et al. 1999; Shields & Hildebrand 1999; Lopes et al. 2002). However, the ultrastructure of antennal sensilla has never been described for any *Neocthena* spp. The purpose of this study was to fill the gap through an anatomic-ultrastructural study of weevil antennal chemosensilla.

According with Bernays & Chapman (1994), all the leaf-feeding insects that have been examined critically have been shown to be able to smell components of the commonly occurring green leaf volatiles such as hexanol or hexenal. It has also been shown that the number and/or sensitivity of receptors is greater for alcohols and aldehydes with six-carbon-atom chain lengths (the major constituents of the called "leaf odor") than for compounds with shorter or longer chains. Because of this general sensitivity, all phytophagous insects probably have the capacity to smell any plant, whether it is a host or not. In addition to these responses to widely occurring plant volatiles, some insects also exhibit sensory responses to the odors of compounds specific to their host plants. As we hypothesize that *Neocthena* weevils are attracted to host-produced volatiles, we addressed the identification and the behavioral activity of water hyacinth volatiles on *Neocthena* weevils.

Therefore, the research work attempted in this paper was motivated by the potential use of water hyacinth volatiles in mass trapping schemes of *Neocthena* weevils and the need of bio-
logical knowledge on the Neoechetina spp. sensory structures as well as the determination of water hyacinth volatiles that mediate the weevil-host attraction.

MATERIAL AND METHODS

**Neoechetina bruchi and N. eichhorniae antennae external morphology**

The description of Neoechetina spp. antennal sensilla terminology and number and placement was realized by using scanning electron microscopy (SEM) at Faculty of Sciences of Lisbon University, Portugal. The weevils were field-collected from water hyacinth plants located in tanks at University of Florida, USDA/ARS Invasive Plant Research Laboratory at Fort Lauderdale, in South Florida (USA), from 1999 to 2001. To examine the types of sensilla, antennae from freshly killed Neoechetina eichhorniae and N. bruchi adults were excised, dehydrated in a graded ethanol series and air-dried. Then, specimens were attached to aluminium stubs with double stick tape, gold coated with a Bio-Polar F 1100 or a Jeol 1500 esputter coater, and viewed with a JEOL JSM 5200 LV scanning electron microscope at accelerating voltage of 15 kV. The types, number and distribution of antennal sensilla were identified from SEM montage micrographs obtained from 3 males and 3 females antennae of each species. Lengths of sensilla were determined by measuring 10 sensilla of each type for both species and sexes.

The sensillar terminology adopted follows that used by Schneider (1964), Dyer and Seabrook (1975), Zacharuk (1980, 1985) and Merriee et al. (1997, 1999, 1999, 2002). Long hair-like sensilla were divided into sensilla chaetica and sensilla trichodea according to the way of attachment to the antennal surface. The hairs standing in a wide flexible joint membrane were classified as sensilla chaetica, but long hairs unmovable at their base when touched were classified as sensilla trichodea. Short pegs were classified as basiconic sensilla.

The statistical analyses included mean comparisons, which were performed using a multiple t-test with Bonferroni probability adjustment procedure. The software package used was Statistica (version 5).

**Attraction of water hyacinth volatiles to Neoechetina bruchi and N. eichhorniae**

Insects. Large quantities of Eichhornia crassipes root material were collected from southern Florida and examined to locate Neoechetina bruchi and N. eichhorniae cocoons, during 1999 to 2001. The cocoons were carefully removed from the roots and held in Petri dishes with moistened filter paper (to keep high humidity) at approximately 28-30°C. Newly emerged adults were collected daily, kept in small vials with filter paper and water hyacinth leaves and their age carefully recorded, until studies were initiated. As weevils younger than 7 days of age were more sensitive to odors than older ones (Weissling et al. 1994) 2-5 days old adults were used for behavioral bioassays.

Plant material and volatile collection. Plant samples were collected from the same locations as the weevils and from aquatic tanks at the University of Florida, USDA/ARS Invasive Plant Research Laboratory at Fort Lauderdale, Florida and consisted of freshly cut *E. crassipes* leaves. Fully expanded fresh leaves (40-50g) were cut into 1cm² pieces with scissors and transferred directly to glass volatiles collection chambers (4.5x30.5cm; Analytical Research Systems, Micanoopy, FL, USA). Aeration were conducted without added moisture by passing filtered air across the leaves and collection of volatiles in collection tubes (7.6x0.6cm) packed with Super-Q adsorbent (30mg; Alltech Associates, Deerfield, IL, USA). Adsorbed volatiles were eluted with CH2O (20µl) then frozen (-20°C) until analyzed by GC-MS. Air was filtered with a series of activated carbon filters (Heat and Mannukon, 1992). Collections were obtained with air flows of 500 ml/min for 18-24 hours with a 12h photoperiod, ~50µl/h and at 25°C. Light was provided by two 100W incandescent bulbs located 30cm above the collection chambers. As a control, volatiles were also simultaneously collected from an identical chamber under the same conditions that lacked plant material.

Volatiles were collected from the headspace of a 5-l flask filled with 300g of chopped water hyacinth leaves. Air was drawn from the flask at 40ml/min for 24h, at 25±0.2°C, over the water hyacinth leaves and through an outlet of Porapak Q (80-100 mesh), 400mg glass tube (6x0.6x5cm) under a vacuum pump. At the end of each adsorption period, the Porapak Q was eluted with solvent (200µl of methylene chloride) dripping into a 2ml glass vial. These solutions of water hyacinth volatiles were used in GC-MS and behavioral bioassays. When not been used, the solutions were stored in the dark at -3°C.

Volatile analysis. The *E. crassipes* foliar constituents were analyzed by GC-MS with an Agilent 6890 instrument fitted with a HP-5MS (Agilent, 30m x 0.25mm, 0.25µm film thickness) HP-5T columns with helium at 36 as a carrier gas. Injections were conducted with an autosampler (HP-7683) split 1:20 at 250°C. The mass selective detector (HP 5973) was heated at 250°C (source) and 150°C (quad) with transfer line 280°C, and ion source filament voltage of 70eV. Component identification was made on the basis of mass spectral fragmentation, retention index with n-paraffins, comparison with authentic constituents, and mass spectral and retention matching. Standards were purchased from commercial sources (e.g., Sigma, St Louis, MO, USA) and were of the highest purity available. Optical isomerism was not investigated.

Behavioral bioassays. Adult weevils were field-collected from south Florida ponds feeding on *E. crassipes* stems and leaves. Weevils were held (27°C; 12h photoperiod) in rearing cages (30x13 cm) and fed fresh *E. crassipes* stems and leaves until tested. As the adults of these weevils are crepuscular, y-tube olfactometer assays were conducted in darkness (red light). All bioassays were conducted in one of three identical Y tubes (Kimble Kontes Vineland, NJ, USA). Each y-tube was composed of a single stem (30cm length x 1cm diameter) joined by two arms (20cm length x 1cm diameter). The y-tubes were oriented horizontally and filtered air (11/min) was directed through each arm of the tube. Each arm was attached to Teflon tubing tosa sample flask (Kimble Kontes Vineland, NJ, USA; 50ml) designated as either a test or a control. Each test sample flask was loaded either with broken *E. crassipes* leaves (5g) or a test compound applied (25µl) to filter paper strips (5x1cm). Test compounds were applied at four concentrations (0.0001, 0.001, 0.01, 0.1mg/µl) in paraffin (Sigma, St Louis, MO, USA). The control flasks were either empty for the broken-leaves test or contained filter paper treated with paraffin (25µl) is a control for the test compounds. Each weevil was introduced individually and a total of 25 weevils of each sex and species was tested. A positive response was recorded when the weevil crawled to within 2cm of the end of either y-tube; a non-response was recorded when the weevil did not reach this point within 15min. After testing each weevil the y-tube orientation was rotated 180° in order to avoid directional factors. Each tube was washed at the end of the day with hot soapy water, rinsed with deionized water, DDH2O (95%), and oven dried (100°C). The two-choice results were analyzed with a G test of independence (SAS-Pc, SAS Institute, Inc, 1999; P = 0.05) after removal of the non-responsive individuals (9%).
RESULTS

**Neochetina bruchi** and *N. eichhorniae* antennae external morphology

**General structure of the antennae**

Both males and females of *Neochetina eichhorniae* and *N. bruchi* have geniculate antennae formed by 11 antennomeres: a long scape (1'), a pedicel (2'), a flagellum consisting of a funiculum (3'-10') and a relatively big club made of 4 subsegments (II-IV clavomeres, i.e. 8th-11th antennomeres) (Fig. 1).

In both species the antennae are sexually dimorphic in length, with females showing a bigger antennae than males: in *Neochetina bruchi*, pedicels measured an average of 1225µm and 1000µm, and the rest of antennomeres 1325µm and 1175µm, respectively in females and males; in *N. eichhorniae*, pedicels measured an average of 1025µm and 800µm and the rest of antennomeres 1125µm and 1150µm, respectively in females and males.

In both sexes and species, the scape and the antennomeres 3 to 5 are almost glabrous, showed only a few sensilla (Fig. 1). An increase in sensilla morphological diversity and number was observed on the distal region of the pedicel and from antennomeres 6 to 7, with the greatest sensilla density (91% in *N. bruchi* and 83% in *N. eichhorniae*) and variety been observed on the club.

**Topography and typology of antennal sensilla**

To simplify description and to preliminarily organize the numerous types, sensilla are grouped on the basis of ultrastructural similarity into 5 types, I to V. Those five different types of sensilla were all found on males and females of *Neochetina bruchi* and *N. eichhorniae*.

![Figure 1: Scanning electron micrograph (SEM) of the antennae of *Neochetina eichhorniae*. The antennae consist proximally of the long scape (Sc) and the pedicel (Pb) and distally of the flagellum (Fr), which in turn is divided into 9 segments. The last four flagellomeres are fused forming the antennal club (Cl). This density of sensilla per antennomere increases distally, being the club densely covered by hairs. The antennae of *N. bruchi* is very similar. Bar: 50 µm.](image-url)

These weevils showed two distinctive types of sensilla, considered here as Type I and Type II. The other three types, considered as sensilla trichodea Type III, sensilla chaetica Type IV) and sensilla basiconica (Type V), were situated on the club only.

**Type 1:** Hair characterized by a tree-like shape, with 2, 3, 4, 5, or 6 branches (subtypes Ia, Ib, Ic, Id, and Ie respectively) (Fig. 2 A, C, D, H). The sensilla range in length from 27 to 47µm. These sensilla are the most numerous type present (62% in females of the two species, 61% and 60% in males, of respectively, *N. bruchi* and *N. eichhorniae*). Type Ia occurs in pedicel, 7th antennomere and club of *Neochetina bruchi*. In *N. eichhorniae* occurs in the same antennomeres plus the 6th. Type Ib occurs in all antennomeres except 3-6 in *N. bruchi*. In *N. eichhorniae* this subtype has the same distribution but appears also on antennomer 3-4 in females and 4 in males. Type Ic occurs in pedicel and antennomere 7 in *N. bruchi*. In *N. eichhorniae* occurs in the same antennomeres plus the 6th. Type Id occurs in pedicel in both species and sexes but shows sexual and specific differences in other antennomeres. In both species occur also in antennomer 7th in females. In *N. eichhorniae* occurs in antennomeres 6-7 in both sexes, plus antennomeres 3-4 in males. Type Ie occurs only in pedicel of both species and sexes.

**Type 2:** Characterized by a "leaf" shape, can be uniform or have 2, 3 or 4 branches (subtypes IIa, IIb, IIc, and IID, respectively) (Fig. 2 A, B, D, H). The sensilla range in length 42 to 62µm. Type IIa occurs in pedicel, antennomeres 6-7 and first two clavomeres (antennomeres 8-9) in both species and sexes. Males of both species show this subtype of sensilla on the scape. Type IIb occurs in pedicel and antennomere 7th in *N. bruchi*. In *N. eichhorniae* this subtype shows a sexual dimorphism, in females appears in pedicel and antennomeres 6-8th, while in males in addition to those, also occur in antennomeres 1-2. Type IIc shows an interspecific distribution. In *N. bruchi* occurs only in the pedicel, but in *N. eichhorniae* appears also in antennomeres 3-4 and 6-7. There are no sexual differences. Type IIId occurs only in the pedicel of *N. bruchi* and males of *N. eichhorniae*. In females of this species also was observed on antennomeres 3-4.

**Type 3:** Sensilla trichodea sensu Zacharkuk (1985), show a hair shaft 42-72µm long, proximally straight and tapering slightly at its ends (Fig. 2 C, D). They were found over the entire club but mainly on the distal region on both males and females on both weevil species.

**Type 4:** Sensilla chaetica sensu Schneider (1964) and Zacharkuk (1980, 1985), show a cuticular apparatus consisting of an outstanding hair shaft 64-80µm long, straight or gently curved, gradually tapering from the base to a blunt tip. They emerge from well-defined sockets and subtend an angle of around 60° with the antennal surface (Figure 2 D, E). These sensilla are relatively few in number but are the longest of all sensilla present. They are distributed symmetrically around the circumference of the club and protude well above all the other sensilla.

**Type 5:** Sensilla basiconica sensu Schneider (1964), with hair shaft 14-18µm long, relatively stout, blunt tipped, peg-shaped, rising on a rigid socket, and having abundant porous thin walls (Fig. 2 F, G). Both species and sexes have this type of sensilla interspersed among the sensilla trichodea, over the club.

**Cuticular pores:** Cuticular circular pits can be found on the club surface, generally associated with sensilla basiconica lying proximally close to the bristle base (Fig. 2 G). These are round, with 0.5-0.6 µm in diameter.
Attraction of water hyacinth volatiles to Neochetina bruchi and N. eichhorniae

Major volatiles extracted from the leaves of water hyacinth

Water hyacinth plants revealed a scarce number of compounds, in extremely low concentrations. The GC-MS analysis of crushed E. crassipes leaves and stems indicated that the major volatile components were (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol, and 2-ethyl-1-hexanol (Fig. 3). These constituents contributed 18, 68, and 2.5% respectively of the total amount integrated.
DISCUSSION

The present study demonstrates that the antennae of Neochetina eichhorniae and N. bruchi contains twelve morphologically distinct sensilla types. In addition, the two species had similar antennal morphology with regard to the number of segments, types of sensilla and their pattern of distribution on the respective segments of the antenna, with very few exceptions.

Types I and II are different from any sensilla reported in other insects: Hylobius abietis (Mustaparta 1973), Curculio cornutus (Hatfield et al. 1976), Hypera meles (Smith et al. 1976), Hypera postica (Bland 1981), Conotrachelus nemerophar (Alm & Hall 1986), Ceutorhynchus assimilis (Isidoro & Solinas 1992), Semidactylus undecimnotata (Jourdan et al. 1995), Agrotes obscurus (Merivee's et al. 1997), Limonius aeruginosus (Merivee's et al. 1998), Pyrophoros chrysoschela (Barlett's et al. 1999), Melanotus vivulus (Merivee's et al. 1999), Manduca sexta (Shields & Hildebrand 1999), Phoracantha semipunctata (Itoes 2000), Lepidoptera sp. (Ben & Mitchell 2001), Bembidion propero (Merivee's et al. 2002). Even extensively reviewed made by Schneider's (1964), Zacharuk (1980, 1985), and Gaino & Rebrova (1999), showed no such type of sensilla.

Serrilla type III, was considered as trichodea. Serrilla similar to these have been described on several species of Curculionidae. They may be identified with Mustaparta's (1973) "type III hairs" of Hylobius abietetis, Alm & Hall's (1976) "hairs type IV" of Conotrachelus nemerophar, Hatfield's (et al. 1976) "sensilla basiconica type II" of Curculio cornutus, Isidoro & Solinas's (1992) "hair-like sensilla" of Ceutorhynchus assimilis. The tubular body at the base of the sensillum is a typical mechanosensitive structure (Zacharuk 1980, 1985), Mustaparta (1973, 1975) on the antennal club of H. abietis, considered that they had either a mechanoreceptive function, or no receptive function, possibly acting as protective hairs. These sensilla, due to their morphological structure, in Neochetina weevils, may have a combined mechanosensory and gustatory function.

Serrilla type IV, considered as chaetica, is very common on weevil's antennae, and may be easily identified with Mustaparta's (1973) "type IV hairs" of Hylobius abietis, Alm & Hall's (1986) "hairs type IV" of Conotrachelus nemerophar, Hatfield's (et al. 1976) "sensilla trichodea type I" of Curculio cornutus, and Isidoro & Solinas's (1992) "sensilla chaetica" of Ceutorhynchus assimilis. Accordingly with Barlett's (et al. 1999) they may have a combined mechanosensory and gustatory function. These sensilla, morphologically typical gustative, have been proven to respond to mechanical stimuli on Limonius aeruginosus (Merivee's et al. 1998). They resemble the one found in the pine weevil (Mustaparta, 1973), "sensillum type IV", which had responses to chemicals in the liquid phase and/or a mechanoreceptive function. Because of their length, in Neochetina weevils, they will be the first to come in contact with the substrate. Antennal tapping of leaf surface, prior to feeding, probably exposes these gustatory sensilla to tactile and chemical stimuli.

Serrilla type V, was considered as sensilla basiconica. Similar sensilla have been reported in several species of Carabidae, Carabidae, Chrysomelidae, Coccinellidae, Curculionidae, Elateridae, and Scarabaeidae, and may be identified with Mustaparta's (1973) "type II hairs" of Hylobius abietis, Alm & Hall's (1986) "hairs type II" of Conotrachelus nemerophar, Hatfield's (et al. 1976) "sensilla basiconica type I" of Curculio cornutus, Bland's (1981) "sensilla basiconica type II" of Hypera postica, Hatfield's (et al. 1976) "sensilla basiconica type I" of Curculio cornutus, Smith's (et al. 1976) "sensilla basiconica type I" of Hypera meles, Isidoro & Solinas (1992) "peg-like sensilla" of Ceutorhynchus assimilis, Jourdan's (et al. 1995) "sensilla basiconica" of Semidactylus undecimnotata, Merivee's (et al. 1997) "sensilla basiconica" of Agrotes obscurus, Merivee's (et al. 1998) "sensilla basiconica" of Limonius aeruginosus, Bartlet's (et al. 1999) "sensilla basiconica" of Pyrophoros chrysoschela, Merivee's (et al. 1999) "sensilla basiconica type I" of Melanotus vivulus, Lopes's (et al. 2002) "sensilla basiconica type I" of Phoracantha semipunctata, and Merivee's (et al. 2002) "sensilla basiconica" of Bembidion propero.

Although no histological studies of N. eichhorniae and N. bruchi antennal sensilla were performed, sensilla basiconica were noted to break off or contact inwardly along their shafts during mounting, demonstrating their thin-walled nature. Comparatively, sensilla chaetica and sensilla trichodea were usually found to be intact. Complementary, the hair distribution pattern is such that they are protected from mechanical damage by the longer sensilla trichodea and chaetica, also found on N. abietis (Mustaparta 1973) and N. meles (Smith et al. 1976). In electrophysiological and behavioral experiments these type of sensilla have been proven to function as olfactory chemoreceptors in N. abietis (Mustaparta 1975), L. aeruginosus (Merivee's et al. 1998), and Phoracantha semipunctata (Lopes et al. 2002). The similarity in location and ultrastructural features of such classical sensilla basiconica, such as a non-flexible base, and a thin, multilayered cuticular wall, which is typical of insect olfactory receptors, with the ability to perceive air-borne stimuli (Schneider 1964; Zacharuk 1980, 1985), allows the assumption that these sensilla are the most probable candidate chemoreceptors on the antenna of N. eichhorniae and N. bruchi.

No interior sensilla were observed in the pets located on the club surface, generally associated with sensilla basiconica, which indicates they may be epidermal gland ducts. The occurrence of this type of pores is common in other Coleoptera, for example, C. nemurpin Alm & Hall's (1986).

Sex pheromone detection is generally attributed to sensilla types disproportionately present in males, for example, the sexual dimorphism in sensillar distribution in Diatresca virgifera (Barlett et al. 1995). The difference in number of sensilla trichodea and basiconica per antenna may be related to their localization and length. There are 2 times (in N. bruchi) and 2.5 times (in N. eichhorniae) as many of the former as of the latter. On the other hand, the short pegs of sensilla basiconica with the relatively thin walls are more protected by the hair type I, and thereby also less exposed to the air stream than sensilla trichodea. Owing to their length and position the later are more exposed, which could enable them to adsorb odorous molecules from lower concentrations, than sensilla basiconica. That's why sensilla trichodea are usually sex pheromones receptors in insects (Merivee 1992; Merivee et al. 1998). In N. eichhorniae and N. bruchi, however, sexual dimorphism is characterized by slightly differences on the location of type II sensilla on scape. On the other hand, our speculations on the functional mode of each sensillum are correct, about 27% and 25% of the antennal sensilla of N. bruchi and N. eichhorniae, respectively, are olfactory, Of these, the majority are probably responsive to host-plant volatiles. Evidence collected so far by the author suggests that there is nc intraspecific chemical communication, e.g. sexual or aggregation pheromones, involved in mate location.

These observations together with the behavioral studies have demonstrated that these weevils are primarily responsive to host-plant volatiles. Despite its global distribution, Eichhornia crassipes is attacked by few phytophagous generalists. This might indicate that water hyacinth leaves have some deterrents for most species. On the other hand, Neochetina bruchi and N. eichhorniae not only successfully feed and reproduce on the weed, as also reveal a strong attraction to young leaves (De Fose & Perkins 1977; Center & Wright 1991), they might have co-evolved with the host plant in relation to semichemicals.
FINAL REMARKS

Although there are a number of effective ways to control water hyacinth, it remains as the world’s most invasive and damaging aquatic plant. Compared with other methods, biological control is more flexible in its application and is environmentally safe. Furthermore, expenditure ceases after the first few years, but the control achieved continues indefinitely. Biological control using insect natural enemies (e.g., Neochetina bruchi and N. eichhorniae) which feed only on water hyacinth can effectively control the weed in some areas. However, these insects do not provide a complete solution. Each water body should be considered separately; an ideal combination of measures should be devised for each water body, depending on many factors and in close consultation with all users of the water. In situations where tolerance of the weed is low (e.g., boat docking sites, irrigation and hydroelectric dams), there is a need for a control technology, which combines the environmental safety of biological control with the speed of chemical or mechanical control. Chemical control may be necessary as an extreme measure, for the rapid destruction of large masses of weed, which are seriously impeding access or navigation. All the larvae of Neochetina spp. and many adults on the sprayed plants are likely to be lost as a result of complete kill of the weed. This should be considered in deciding the areas to be treated. In addition to the possible problems from desiccation when the weed is decomposing. Where Neochetina spp. are being introduced, any herbicide treatment should of course be kept well away from the introduction points.

In order to try to speed the effects of biological control agents, augmentative releases of Neochetina spp. have been suggested. If large numbers of weevils could be harvested from undisturbed areas or areas targeted for herbicide treatment or held in designated water hyacinth infested areas for redistribution at a later time and then applied to inipient plants or introduced in high densities on seeded water hyacinth, the weed population explosion would be suppressed reducing expenditure of money, time, and risk of environmental contamination. Motivated by the possible use of mass trapping schemes to optimize the control of water hyacinth by Neochetina bruchi and N. eichhorniae, an attempt to understand the weevil-host attraction was presented.

The study of the antennal fine structure of Neochetina spp. revealed twelve morphologically distinct sensillum types, aggregated into 5 main types. Type I, different from sensilla found in other insects, and the most abundant in Neochetina weevils, was found on almost all antennal segments. By contrast, type II, also a unique feature, was restricted to very few antennomeres. Comparisons of the cuticular specialization, ultrastructure, and location of these sensillae to those described by other authors suggest that these sensilla are capable of responding to various stimuli, viz. tactile as well as thermo — and/or hygrosensation. The distribution patterns of other three sensillar types provided evidence for the importance of the club of Neochetina bruchi and N. eichhorniae on host-plant reception. All these sensory structures are situated on the antennal club, i.e. 8-11th antennomeres, and consist of three types of chemosensilla, namely sensilla basiconica, chaetica, and trichodea. Sensilla basiconica are ultrastructurally typical olfactory, comparatively most suitable to detect environmental volatiles present in relatively high concentrations, such as host-plant odours. Sensilla chaetica, externally represented by the most projecting setae from the antennal surface, are ultrastructurally typical contact chemoreceptors strategically located to easy touch and taste host-plant surface. Sensilla trichodea, the second most numerous chemosensilla, distinctly sicle-shaped; simplest chemoreceptors are ultrastructurally typical olfactory of moderate efficiency, suitable for detecting environmental volatiles present in low concentrations such as pheromones. Therefore, sensilla basiconica and sensilla trichodea are...
candidate chemoreceptors based on their close resemblance to sensilla reported to have this function in other insects. However, electrophysiological investigations using single-celled recording are needed to confirm their functions. The number of sensilla on the antennae of *N. enhorimie* and *N. bruchii* is not as high as in other weevils. It thus appears reasonable to conclude that a non-polyphagous species has a narrower fit for the neural template coding for feeding behavior than polyphagous feeders. Consequently, a behavioral sequence may only be elicited if a series of peripheral signals indicate a perfect match for the template for feeding behavior.

To determine whether *Eichornia* cressipes odors play a role in host-finding behavior of *Neochetina* spp., identification of the weed volatiles that elicited behavioral weevil responses was also object of research. The results indicate that the primary volatile produced by crushed *E. cressipes* stems and leaves is L-3-hexen-1-ol and this constituent is attractive to both species and sexes of the biological control agents. Additional attractant volatiles may be found that are species-specific and that separate these two weevil species in the field. These volatiles may be additional plant-or weevil-produced compounds that have yet to be elucidated.

As there is no evidence supporting the existence of sexual or aggregation pheromones, or any non-chemical long-range intraspecific communication mechanism (e.g. sound), it is not surprising that both sexes exhibit similar olfactory capabilities. However, the weak attraction revealed to water-hyacinth volatiles, might suggest a stronger importance of visual rather than visual clues on host-plant detection. The weevil can determine whether the host tissue is of good quality in terms of nutritional and moisture factors. Possibly, it must not only determine that the plant underneath is the proper host and is suitable for reproduction, but it must also judge potential competition by whether nearby areas have other weevils beginning their attacks. The host-specificity of both species has been demonstrated during extensive host testing and confirmed by observations after their release in numerous countries. Despite being released widely there are no reports of these weevils seeking out and damaging plants other than *E. cressipes*. Further support for their specificity comes from knowledge of *Neochetina* spp. life-history.

In light of all this evidence, releases of these weevils into new countries can now be carried out with very few hosts testing. For Portugal, for instance, we suggest only further testing, including both multiple choice and no choice oviposition, larval development and adult feeding, on *Aplius grovei*, *J. auratum*, *Phascolus lunatus* and *Pyrus domestica*, to which are no available data, and constitute important agricultural cultures in the country.

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ABORDAGENS ECOTOXICOLÓGICAS PARA AVALIAÇÃO DE EFEITOS SECUNDÁRIOS DE HERBICIDAS

Ecotoxicological tools to evaluate environmental side-effects of herbicides

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RESUMO
Os herbicidas podem atingir os ecossistemas aquáticos envolvidos. Sendo assim, os potenciais riscos ambientais relacionados com a utilização de herbicidas necessitam de ser avaliados antes (prospectivamente), bem como após (retrospectivamente) a sua colocação no mercado. No presente estudo, são discutidas três ferramentas ecotoxicológicas actualmente usadas para a avaliação do risco de herbicidas: i) testes de toxicidade laboratorial; ii) distribuição da sensibilidade de espécies (DSE); e iii) modelos de ecossistemas. Estes testes de toxicidade laboratoriais são úteis para obter efeitos secundários dos pesticidas sobre organismos individuais de diferentes níveis tróficos com reprodutibilidade e precisão experimental, podendo ainda ser utilizados em amostras de campo para monitorizar a qualidade da água. As curvas da DSE fornecem uma estimativa mais realista da susceptibilidade da comunidade aquática aos efeitos de herbicidas, pois incorporam um elevado número de organismos. Os modelos de ecossistemas, apesar de serem mais dispensiosos, permitem a avaliação ecotoxicológica com maior realismo ecológico.

Palavras-chave: Efeitos secundários ambientais, ecotoxicologia aquática, pesticidas

ABSTRACT
Herbicides applied to crops for weed control may enter aquatic ecosystems surrounding agricultural fields. Subsequently, potential environmental risks related with the use of herbicides need to be assessed before (prospectively) as well as after (retrospectively) the pesticide is authorized for use. In the present study, three ecotoxicological tools currently in use for the risk evaluation of herbicides are discussed: i) laboratory toxicity tests; ii) species sensitivity distributions (SSDs); and iii) model ecosystems. Laboratory bioassays are especially useful to obtain a true experimental reproducibility and precision of the effects of pesticide stress on single organisms, and are also suitable using field samples to monitor water quality. However, establishing causal relationships between herbicide exposure and environmental side-effects is often hampered because of obscuring variables in the field. SSDs incorporate a greater number of test organisms and therefore provide a better estimation of the susceptibility of the aquatic species assemblages to herbicide stress. Model ecosystem studies allow the evaluation of causal relationships between exposure and effects under fairly ecologically realistic conditions. On the other hand, they are more costly and labour intensive as compared to the other test methods.

Key-words: environmental side-effects, aquatic ecotoxicology, pesticides