UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

EFFECTS OF SELECTED NEUROTOXIC INSECTICIDES ON VIBRATIONAL COMMUNICATION OF THE SOUTHERN GREEN STINK BUG (*Nezara viridula*, Heteroptera: Pentatomidae)

DISSERTATION

Alenka Žunič

Mentor: prof. dr. Andrej Čokl

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Look deep into nature, and then you will understand everything better
(by Albert Einstein)

SUMMARY

Modern agriculture depends on the extensive use of insecticides. Serious questions have been raised about their non-target effects on human health, terrestrial and aquatic wildlife. Lethal toxicity of insecticides on non-target arthropod species has been studied thoroughly. However, sublethal effects of insecticides, which may not kill the animals but have been shown to disrupt the behaviour, are poorly understood. In the present study, the sublethal effects of two widely used insecticides (deltamethrin and imidacloprid) on the substrate-borne (vibrational) communication of the *Nezara viridula* males were evaluated. More specifically, we evaluated the ability of treated males to recognize and respond to female vibrational signal that plays a crucial role in the species recognition and mating behaviour. In addition we tested the effects of deltamethrin and imidacloprid on the reproductive success of *N. viridula* males.

After topical exposure of males to sublethal doses of deltamethrin and imidacloprid, playback experiments were performed. Males were placed on the loudspeaker membrane and stimulated with the synthesised and the natural female calling songs (FCS). Their vibrational responses were recorded by a laser vibrometer and the quality of their responses was analysed at different time intervals within 72 hours after the exposure to insecticides. Additionally, dose-dependent response was assessed for imidacloprid.

Deltamethrin and imidacloprid treatment decreased the general responsiveness of males however, they differed in the time-course of their action. Deltamethrin induced rapid and strong decrease in responsiveness, with full recovery of the intoxicated males after 48 hours. Imidacloprid, on the other hand, induced prolonged toxicity and decreased responsiveness with low ability to recover within the tested time period. Furthermore, imidacloprid had greater negative effect on the nature of the response and tolerance for different FCS parameter values. These differences could be associated with specific insecticide type, different target sites and mode of actions at the neuronal level.

Preliminary results of the present study suggest negative sublethal effects of deltamethrin and imidacloprid on reproductive success of *N. viridula*, but further studies are needed to get clear interpretation of the results.

POVZETEK

Sodobno kmetijstvo uporablja vedno več insekticidov, ki predstavljajo grožnjo za potrošnike, netarčne žive organizme ter obremenjujejo okolje. Učinki letalnih doz insekticidov so dobro znani in raziskani. Posledice subletalnih doz insekticidov, ki živali ne ubijejo ampak imajo lahko negativne učinke na vedenje, so slabše raziskane. V disertaciji smo raziskovali subletalne učinke dveh insekticidov (deltametrina in imidakloprida) na komunikacijo preko podlage (vibracijska komunikacija) pri samcih zelene smrdljivke (*Nezara viridula* L., Heteroptera). Vibracijski signali imajo ključno vlogo pri razmnoževalnem procesu zelene smrdljivke, omogočajo vrstno prepoznavanje in določanje položaja partnerjev v prostoru. V disertaciji smo ugotavljali v kolikšni meri insekticidi vplivajo na samce in njihovo sposobnost prepoznavanja in odgovarjanja na vibracijske signale samice. Preučili smo tudi vpliv deltametrina in imidakloprida na uspešnost razmnoževanja tretiranih samcev.

Po izpostavitvi samcev subletalnim dozam deltametrina ali imidakloprida smo jim na zvočniku predvajali sintetični in naravni pozivni napev samice (female calling song = FCS). Vibracijske odzive samcev v različnih časovnih intervalih do 72 ur po aplikaciji insekticida smo posneli z laserskim vibrometrom in jih analizirali. Za imidakloprid smo dodatno preučili tudi vplive različnih subletalnih doz.

Deltametrin in imidakloprid sta znižala splošno odzivnost samcev, vendar se je časovni potek učinkov med insekticidoma razlikoval. Deltametrin je povzročil hiter in močan upad v odzivnosti samcev, ki mu je sledilo popolno okrevanje samcev, 48 ur po izpostavitvi. Imidakloprid je izzval dolgoročno znižanje odzivnosti pri samcih z zelo nizko sposobnostjo okrevanja znotraj 72 ur po izpostavitvi. Izpostavitev imidaklopridu je imela tudi negativne posledice na samčevo sposobnost prepoznavanja in odgovarjanja na FCS s spremenjenimi lastnostmi. Razlike v učinkih obeh preučevanih insekticidov so lahko posledica tipa insekticida ter razlik v tarčnih mestih in načinu delovanja na živčnem nivoju živali.

Rezultati predhodne raziskave o subletalnih vplivih deltametrina in imidakloprida na uspešnost razmnoževanja zelene smrdljivke, so pokazali negativne učinke, vendar so za natančnejšo interpretacijo učinkov insekticidov potrebne še nadaljne raziskave.

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1 INTRODUCTION

1.1 Problem description and relevance

This doctoral work combines the research of the process of intraspecific substrate-borne (vibrational) communication in the southern green stink bug, *Nezara viridula* L. (Heteroptera: Pentatomidae) (Figure 1), and the investigation of possible effects of sublethal doses of neurotoxic insecticides on the selected behavioural pattern.

Figure 1: Male of the southern green stink bug (*Nezara viridula* L.) on a bean leaf.

Insects represent a crucial part of the food chain and are indispensable as pollinators. However, insects destroy approximately one third of globally produced food and are vectors for several serious diseases. Modern agriculture and plant protection strategies depend heavily on the extensive use of insecticides. Serious questions are raised about their potential harmful, non-target effects on human health, terrestrial and aquatic organisms. Many studies on lethal, acute toxicity of insecticide on non-target species have been performed. On the other hand, sublethal doses of insecticides may not kill the insect, but are known to disturb behavioural patterns like reproduction, host finding, feeding behaviour and locomotion, all of which lead to disorder in population dynamics (Colin, 1999). Non-target organisms may encounter insecticides by intercepting sprayed droplets and via contact with insecticide residues on treated substrates (Al-Deeb et al., 2001). The loss of great amounts of bees, as typical non-target organism, has been recorded in the last few years and has been hypothetically connected with incorrect and uncontrolled use of insecticides. Non-

target effects of insecticides can also have large negative effects on predator/prey and host-parasitoids systems of integrated pest management (IPM) strategies (Desenux et al., 2006, Walker et al., 2007). Several studies on non-target effects of insecticides were conducted, mainly on pollinators and predatory fauna (Desneux et al., 2007).

Understanding the mode of action of sublethal insecticide doses and their effectiveness in altering behaviour of insects can contribute to reduction of the use of insecticides in pest management practice. Strategies promoting judicious use of insecticides are more compatible with low-input agriculture and IPM systems, which minimize detrimental effects on beneficial and other non-target organisms.

Communication is an important and often conspicuous part of the behavioural repertoire of animals (McGregor, 2005). It is a crucial part of social behaviour and therefore the basis that determines most aspects of reproduction and survival. In many insect species vibrational communication is an important part of social and ecological interactions (reviewed in Čokl and Virant-Doberlet, 2003, Virant-Doberlet and Čokl, 2004, Cocroft and Rodriguez, 2005). It plays an important role in the mate recognition system and enables a male to find a female in their environment (Ota and Čokl, 1991, Čokl et al., 1999, Miklas et al., 2003a).

Nezara viridula, the southeren green stink bug, is one of the best investigated plant feeding penatomid species. Due to its well investigated biology, relatively simple laboratory rearing, well investigated behaviour, morphology and function of receptors and underlying neuronal network, its polyphagous nature, worldwide distribution and its pest status, *N. viridula* is a most suitable test organism for assessing sublethal effects of neurotoxic agents on vibrational communication. This research could provide new knowledge on strategies for control of populations of *N. viridula*, which is globally one of the economically most important pest species (Panizzi et al., 2000) invading new areas in Slovenia and Europe due to long-term warming of the atmosphere. In addition, results obtained by investigating the herbaceous pentatomid *N. viridula*, will help to understand insecticide risk for the predatory species of the same family Pentatomidae, which are known to also use

vibrational communication as a part of their mating behaviour (Gogala, 2006, Žunič et al., 2008) and are important biological control agents (De Clercq, 2000).

Organophosphates (dicrotophos, methyl parathion, acephate), pyrethroids (cyfluthrin, bifenthrin, lambda-cyhalthrin, deltamethrin) and neonicotinoids (thiametoxam, acetamiprid, imidacloprid) are used to control plant feeding stink bugs (Tillman, 2006). Oral and residual toxicity (evaluating mortality rate, fecundity perturbation, locomotory and feeding disorders) of this insecticide to stink bugs has already been demonstrated (De Cook et al., 1996, Elzen, 2001, Tillman and Mullinix, 2004, Vandekerkhove and De Clercq, 2004, Snodgrass et al., 2005, Tillman, 2006). To our knowledge, nothing is known about the influence of these insecticides on the behavioural pattern such as vibrational communication.

For the purpose of this study deltamethrin (a pyrethroid) and imidacloprid (a neonicotinoid) were selected as two most widely used insecticides also applied to crops known to be attacked by *N. viridula*.

1.2 The aims of the present study

The aims of the present study are:
(1.) to determine the male preference and selectivity for the female calling song (FCS) parameters, i.e. to what extend FCS is employed in the species recognition system and to identify the recognition mechanism used by the males;
(2.) to examine the effects of different sublethal doses of neurotoxic insecticides on the vibrational communication of *N. viridula* males, focusing on species recognition, the ability of males to respond to the FCS and to discuss the relation between behavioural effects and effects on the nervous system of insects at peripheral and central level;
(3). to examine the sublethal effects of insecticides on the reproductional success of *N. viridula* males.

In order to get results we carried out the following four experiments:

- Experiment 1: we analysed vibrational responses of *N. virdula* males to synthesised female calling song of different temporal and frequency characteristics. We investigated the characteristics of the FCS that are necessary for eliciting male response and the informational value of specific FCS parameter for its recognition. We determined male preference and selectivity for five FCS parameters independently: pulse train duration, interval between pulse trains, duty cycle, repetition time and dominant frequency of the pulse train.
- Experiment 2: we analysed the effects of sublethal doses of imidacloprid and deltamethrin on the general responsiveness of males to the FCS stimulation and the quality of males response in relation to different insecticides, different doses of treatment and time interval after treatment.
- Experiment 3: we tested the effects of the insecticides on the preference and tolerance of males for different values of two FCS parameters (pulse train duration and dominant frequency).

In the experiments 1, 2 and 3, playback trials were carried out. Males were stimulated with stimuli of variated parameter values (outside the range characteristic of the natural FCS) and with stimuli of values characteristic of the natural FCS. Their responses were recorded and analysed.

- Experiment 4: we tested the effects of imidacloprid and deltamethrin on the reproductive success of the *N. viridula* males. Treated males were paired with untreated females and their copulatory success, fecundity and fertility was followed.

1.3 Hypothesis

Neurotoxic insecticides influence behaviour mediated by neurons, including vibrational communication and mate recognition system. Song recognition is enabled by precise coding and filtering processes at the receptor and central nervous level.

We hypothesise that:

1) FCS parameters play an important role in the species recognition system in *N. viridula*;
2) the insecticide decreases the sensitivity of receptors and/or decrease the ability of higher order neurons to extract the information from communicational (substrate-borne) signals, resulting in a decrease of the general responsiveness of males to FCS stimulation;
3) by analysing male response to frequency playback tests, we can evaluate the effects of the neurotoxic agent at the receptor level;
4) analyses of the quality of male response and changes in preference for temporal parameters of FCS can indicate the effects on the central neuron level and the motor networks that generate and control the response;
5) insecticide influences the reproduction success of treated males;

2 THEORETICAL BACKGROUND

2.1 Neurotoxic insecticides

Insecticides are chemical or biological agents used for controlling insect populations. Control is achieved by killing the insect or otherwise preventing it from engaging in normal behavioural patterns. According to Ware and Whitacre (2004) some 10,000 of more than 1 million species of insects are crop-eating, and of these, approximately 700 species cause most of the insect damage to man's crops worldwide, both in the field and in storage. Advances in understanding the biology of pests undoubtedly enhanced the utility of pesticides beginning in the 19th century. At the beginning of World War II (1940), the insecticide selection was limited to several arsenicals, petroleum oils, nicotine, pyrethrum, rotenone, sulphur, hydrogen cyanide gas and cryolite. The World War II opened the "Modern era of chemical control" with the introduction of a new concept of insect control-synthetic organic insecticides, the first of which was DDT (Ware and Whitacre, 2004). Organochlorines, organophosphates, methylcarbamates and pyrethroids are organic insecticides following the first generation. Relatively new and small group (six active substances) of insecticides are neonicotinoids and are now replacing organophosphates and carbamates. Chemical insecticides have been used successfully for many decades. They are likely to remain as a major contributing factor for reducing the risk of insect-borne diseases and enabling a productive agriculture for many years to come. Along with the introduction of new crop varieties and mechanisation, synthetic chemicals are largely responsible for the doubling of crop yields per hectare since 1940 (National Research Council, 1989). However, the benefit of agricultural chemicals comes with some risks. Risks include chemical contamination of ground water and running waters, effects on non-target organisms, risk posed to human health from acute and chronic exposure to insecticides, elimination of natural enemies causing secondary pest breaks and development of resistance to chemicals (Shieh, 1994).

Neurotoxicity refers to the capability of inducing adverse effects in the central nervous system, peripheral nerves and/or sensor organs. A chemical is considered to be neurotoxic, if it is capable of inducing a consistent pattern of neural dysfunction or change in the chemistry or structure of the nervous system. In most basic terms, the neurological effects of insecticides are categorised as either neuroexcitatory or neuroinhibitory. At the level of a whole organism the observable behaviour effects of neuroexcitation are hyperactivity, tremors, and rigid paralysis, while neuroinhibition results in immobility and flaccid paralysis. The effects of neuroexcitation generally occur rapidly, while those of neuroinhibition take longer to become apparent. Target sites and modes of action are very important insecticide characteristics. Target sites are defined as the specific bio-chemical or physiological sites within an organism, that insecticide molecules interact with to create toxic effects. The physical and chemical properties (e.g. Hill coefficient) of insecticides determine the target sites, which they will interact with. Target sites of the most prevalent neurotoxic insecticides in use worldwide are: acetylcholinesterase enzyme, voltage-gated sodium channels, GABA and glutamate-gated chloride channels and nicotinic acetylcholine receptors. Modes of action of insecticides at these sites are diverse and range from enzyme inhibition, to receptor agonism (stimulation), receptor antagonism (blockage) and ion channel modulation.

Understanding the activity of neurotoxicant on the nervous system may help to explain the nature of their possible sublethal behavioural effects. On the other hand, the behavioural symptoms of sublethal poisoning may help to elucidate their mode of action. Understanding the mode of action of currently available insecticides will help to determine the chemical properties of novel compounds that may be ideally suited for modification of insect behaviour at doses that can be used safely and economically in agriculture (Haynes, 1988).

2.1.1 Pyrethroids

Pyrethroids are a class of synthetic insecticides that have been designed and optimized based on the structures of the pyrethrins, the six insecticidal constituents

of the natural insecticide pyrethrum found in flowers of *Chrysanthemum cinerariaefolium* Vis. (Nauman, 1990). Pyrethroids have been produced since 1940 and from 1970s; they have been widely used to control insect pests in agriculture and in home (Elliot, 1980). They are more stable in light and air than natural pyrethrins. The main advantages of pyrethroids are high insecticidal potency and low mammalian toxicity. They have a highly nonpolar nature, low mobility in soil and are adsorbed strongly to the sediments of natural water systems. Despite the high attachment of pyrethroids to living organisms, their capability to bioconcentrate is mitigated by their metabolism and subsequent elimination (Laskowski, 2002). There are several ways for a pyrethroid to enter the body of an insect: nonstereospecific and rapid penetration through the cuticle of the body or tarsi, via the vapour phase together with oxygen supply, through oral uptake and digestion or penetration through the wall of the intestines into the hemolymph.

The known pyrethroids are classified in to two groups according to their structure and different effects at nerves in vivo and in vitro (Nauman, 1990). Type I pyrethroids are devoid of a cyano moiety at the α position (i.e. permethrin), while type II pyrethroid have an α-cyano moiety (i.e. cypermethrin and deltamethrin). Type I are more effective in vitro-on nerve preparation, on the other hand type II are more toxic in vivo-more insect-toxic. Type I poisoning symptoms are characterized by hyperexcitation, ataxia, whole-body tremor, sensitivity to external stimuli. Pyrethroids of type II cause hypersensitivity, lack of coordination, and/or choreoathetosis (sinuous writhing convulsions paralysis). Pyrethroids are known to alter normal function of insect nerves by modifying the kinetics of voltage – sensitive membrane channels.

Membrane bound ion channels mediate the exchange of information between the outer environment and the information processing within the central nervous system (CNS). Action potential travelling along the axon must be transmitted across the synaptic cleft to be propagated further. The mechanism of synaptic transmission is well understood (Hammond, 1996, Stenersen, 2004). The nerve cell membrane at resting potential has an excess of positive charges on the outside and an excess of the negative charges on the inside of the nerve cell. The separation of charges is maintained because the membrane is relatively impermeable to sodium ions (Na^+),

more or less open to chloride ions (Cl^-) and has regulated permeability to potassium ions (K^+), resulting in high concentration of K^+ on the inside and a high concentration of Na^+ on the outside of the cell membrane. Potassium diffusing down the potassium concentration gradient and creates a negative-inside membrane potential. This build up of charge leads to potential difference across the membrane. A local membrane depolarization caused by an excitatory stimulus causes some voltage-gated sodium channels in the neuron cell surface membrane to open. Sodium ions diffuse in the cell through the channels along their electrochemical gradient. The influx of positive charge (Na^+) cause a reversal in the potential difference across the membrane from negative- to positive-inside, it depolarizes the cell membrane. As Na^+ enter and the membrane potential becomes less negative, more sodium channels open, causing an even greater influx of Na^+. As more sodium channels open, the sodium current dominates over the potassium leak current and the membrane potential becomes positive inside. Once a membrane potential reaches values around +40 mV, voltage-sensitive inactivation gates of the sodium channels, sensitive to the now positive membrane potential gradient, close and further influx of Na^+ is prevented. The opening of the sodium channels leads not only to a decrease and reversal of the electrical potential difference at the site of the open channel, but also to a voltage drop further down the axon, causing the sodium channels at that point to open and the signal propagates further down the axon. The sodium channels inactivate after a very short time. During this period (as the cell membrane becomes more positive), the voltage-gated potassium channels open and K^+ flow outward, which brings the membrane potential to a more negative-inside, repolarisation and restoring the membrane to the resting potential occurs. The large outward current of K^+ through the voltage-gated potassium channels causes the temporary overshoot of the electrical gradient, with the inside of the neuron being even more negative (relative to the outside) than the usual resting potential. This is called hyperpolarisation. The voltage-sensitive inactivation gates on the potassium channels now close and the continual movement of K^+ through potassium leak channels again dominates the membrane potential. Sodium-potassium pumps continue to pump Na^+ out and K^+ in preventing any long-term loss of the ion gradients. The resting potential of -70 mV is re-established and the neuron is said to be repolarised.

Pyrethroids slow the kinetics of both opening and closing of the sodium voltage-gated channels resulting in delayed or/and prolonged openings. These insecticides exhibit a negative after potential, indicating that the axon does not readily recover to its resting stage. They also cause repetitive discharge of axonal action potential in response to a single stimulus, therefore neuron can be readily excited again. Pyrethroids can also cause slow depolarization of nerve membrane, which reduces the amplitude of the action potential, leading to a loss of electrical excitability.

Deltamethrin

Deltamethrin (Table 1, Figure 2,) as one of the most potent of the fourth generation synthetic pyrethroids is widely used to protect crops against a variety of pest insects (Nauman, 1990, Pawlisz et al., 1998, Desneux et al., 2006). Deltamethrin kills insects by contact and through digestion. It was developed in 1974 (Elliot et al., 1974). In 2007, deltamethrin was registered in more than 100 countries worldwide, among them in Argentina, Brazil, Canada, China, France, Germany, India, Indonesia, Italy, Pakistan, Spain and USA. The active ingredient deltamethrin is found in a variety of commercial insecticide products with different trade names, such as Butoflin, Butoss, Butox, Cislin, Crackdown, Cresus, Decis, Decis-Prime, K-Othrin, and K-Otek. They are used to control apple and pear suckers, plum fruit moth, caterpillars on brassicas, aphids and moths on glasshouse cucumbers, tomatoes, peppers, potted plants, ornamentals and field crops. Formulations include emulsifiable concentrate, wettable powders, ultra low volume and flowable formulations and granules. The reasons for wide use of deltamethrin are its insecticidal potency, photostability persistence and its efficiency at very low dosage (Nauman, 1990). The toxicity of an insecticide to an organism is usually expressed in terms of LD_{50} (lethal dose, this value represents the dose per unit weight lethal to 50% of a population of test animals). LD_{50} of deltamethrin is 10 to 100 fold lower from the LD_{50} of other pyrethroids (e.g. cypermethrin, fenvalerate, permethrin).

Deltamethrin is classified as the type II pyrethroid. Studies on cockroaches have shown that it begins to depolarize the axonal membrane within 5 min after the application (Laufer et al., 1985). It does not initially modify the shape of the action

potentials, but it inhibits sodium channel inactivation. It enables prolonged sodium current into the cell, which leads to a sustained membrane depolarization and eventually to a conduction block. Opening and closing of the sodium channels normally occur in less than a millisecond during passage of the action potential. Deltamethrin prolongs the mean opening time by 8–10 fold and the sodium leaks out when the channels should be closed, which results in a distinct tail current (Nauman, 1990).

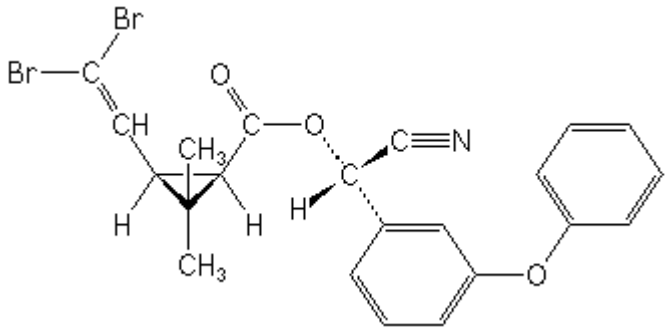

Figure 2: Chemical structures of deltamethrin. (S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate.

Deltamethrin is toxic also to non-target organisms. It has been found to be extremely toxic to fish. The 48 h LC_{50} value of deltamethrin in guppies (*Poecilia reticulata* Peters) was found as 5.13 mg/l (Viran et al., 2003) and for freshwater catfish (*Clarias gariepinus* Burchell) as 40.01 mg/l (Datta and Kaviraj, 2003). Pyrethroids are highly toxic to aquatic invertebrates. The LC_{50} values for most pyrethorids is less than 1 μg/l (Sanchez-Fortun and Barahona, 2005). The changes in aquatic fauna may be followed by the increase of the algae population (NRCC 1986, Tidou et al., 1992). Due to different binding domains of the voltage-sensitive sodium channel in fish, birds and mammals, mammals and birds have been shown to be less sensitive to deltamethrin (Eels et al., 1993).

Earthworms are an important part of the soil ecosystem; they make important contributions to soil fertility and the breakdown of organic material, affect water transport, aeration and soil structure. They are widely distributed, general representatives of the soil fauna, they adapt well to laboratory conditions and are highly sensitive to their surroundings. They are used as a model organism in

terrestrial ecotoxicology. Acute toxicity tests as well as investigations of sublethal effects on their growth and reproduction have been measured (Spurgeon et al., 2003). Among others, deltamethrin has lethal effect (14-day exposure with median LC_{50} of 432.9 mg/kg) on earthworms and inhibits growth and cellulose activity, which can lead to a decrease in their population and to changes in the soil ecosystem (Shi et al., 2007).

Deltamethrin has significant negative effects on non-target forest arthropods, such as spiders, isopods, parasitoids and ground-living polyphagous predators, ants, cantharides, coccinellids and mirids (Schulze et al., 2005, Matcham and Hawkes, 1985, Rodriguez et al., 2003). Laboratory bioassays were carried by Wiles and Jepson (1994b) on a wide range of beneficial invertebrates (predators and parasitoids) exposed to treated wheat foliage, sandy loam soil, and glass surfaces. The 72 h LD_{50} values varied between 4.2 g a.i./ha and 267 g a.i./ha. LC_{50} values of deltamethrin (24 h, topical application, 3.9 μg (AI)/ml) was reported for parasitoid *Trissolcus grandis* Thompson (Hymenoptera: Scelionidae) by Saber et al. (2005). Deltamethrin was proved highly toxic to honeybees, with median LD_{50} of 25 ng/bee determined in the laboratory (Atkins et al., 1981, Badiou et al., 2008).

Table 1: Physical and chemical properties of selected insecticides. A pyrethroid insecticide-deltamethrin and a neonicotinoid – imidacloprid (taken from: Laskowski, 2002, Scorza et al., 2004 and Fossen, 2006.

Property	Deltamethrin	Imidacloprid
Molecular weight, g/mol	505.2	255.7
Colour	Colourless	colourless
Physical state	Crystal	crystal
Melting point, ˚C	101-102	144
Density, g/cm^3 at 25˚C	0.55	1.54
Odour	Odourless	odourless
Solubility in water (mg/l)	0,002 (20 ˚C)	610 (20 ˚C)
Solubility in organic solvents (g/l)		
isopropanol	0.6 (20 ˚C)	1.2 (20 ˚C)
toluene	250 (20 ˚C)	0.68 (20 ˚C)
Octanol-Water coefficient Log K_{ow}	6.1 (25˚C)	3.27 (21˚C)
Soil Sorption Coefficient K_{oc} (ml/g)	6921 (20ºC)	132-310 (20ºC)
Vapour pressure, mmHg at	$1.5x10^{-8}$ (25˚C)	1.00×10^{-7} (20ºC)
Henry's law constant (atmm^3/mol)	$1.2x10^{-4}$ (25˚C)	$6.5x10^{-11}$ (20˚C)
Soil half life (days)	13 (25˚C)	85 (25˚C)

2.1.2 Neonicotionids

Neonicotinoids are the most important new class of synthetic insecticides of the past three decades. Their worldwide annual sales of around US$1 billion, accounting for nearly 15% of the global insecticide market (Tomizawa and Casida, 2005). They are increasingly used for systemic control of aphids, leafhoppers, whiteflies and other plant sucking insects due to an excellent plant-mobile systemic property conferred by moderate water solubility. Neonicotinoids replaced the organophosphorus compounds and methylcarbamates, of which effectiveness decreased due to resistance development and/or increased restrictions due to toxicological considerations. Although crop protection is major use for neonicotinoids, pest insect control on pets or companion animal is also a significant market (Tomizawa and Casida, 2003).

The neonicotinoids are polar, non-ionized and biodegradable, therefore they do not accumulate in mammals or through food chain. Their polar nature makes them potentially mobile in soil but this is mitigated by their low rate of application. Fast degradation of imidacloprid adds to the fact that imidacloprid does not represent high risk for groundwater contamination. Photostability is an important factor in field performance of the neonicotinoids (Tomizawa and Casida, 2003). Neonicotinoids such as imidacloprid and thiamethoxam have a chloro-substituted heterocyclic group, joined to a second heterocyclic ring, whereas neonicotinoids such as acetamiprid have an acyclic group. All neonicotinoids have the same mode of action, affecting synaptic neurotransmission in the nervous system.

A common mode of synaptic transmission is found in many synapses in the nervous system of insects and in mammals also in the neuromuscular junction. Acetylcholine (ACh) is one of the neurotransmitters, stored in membrane bounded vesicles. When an action potential reaches the presynaptic membrane, it opens voltage-gated calcium channels and allows calcium ions (Ca^{2+}) to flow into the terminal. The increase in intracellular free Ca^{2+} causes release of ACh from synaptic vesicles into the synaptic cleft. Released ACh diffuses across the synaptic cleft and binds to nicotinic acetylcholine receptors (nAChR) in the postsynaptic membrane. The

neurotransmitter molecule binding to the receptor site results in a brief ionic current through the membrane of the postsynaptic cell. Nicotinic acetylcholine receptors are commonly found in the insect nervous system, on both post- and pre-synaptic nerve terminals, and on the cell bodies of sensory neurons, motor neurons and interneurons (Jeschke and Nauen, 2005). Neonicotinoids penetrate easily into the nervous system (Yu, 2008). In insects, they mimic ACh by acting as agonists and bind to the nAChR. This activation causes an influx of Na^+ and generation of an action potential. Under normal physiological conditions, the synaptic action of ACh is terminated by the enzyme acetylcholinesterase (AChE), which rapidly hydrolyzes the neurotransmitter. Because the insecticide is not hydrolyzed or destroyed by AChE, the persistent activation leads to an over-stimulation of cholinergic synapses, hyperexcitation, convulsion, paralysis, and finally to death of the insect. They act selectively on insect nAChRs, accounting at least in part for the selective toxicity to insects over vertebrates. Neonicotinoids possess either a nitro or a cyano group and these groups have been postulated to contribute directly to their selectivity (Kagabu, 1999, Matsuda et al., 2001, Tomizawa and Casida, 2003, Tomizawa and Casida, 2005).

Imidacloprid

The most commonly used neonicotinoid is imidacloprid (Table 1, Figure 3) also considered to be one of the insecticides used in the largest volume (Cox, 2001). Imidacloprid as the first commercialised neonicotinoid was patented in the 1985 and introduced on the market in the 1991. Since its launch, products containing imidacloprid have been registered in about 120 countries and are marketed for use on over 140 agricultural crops. Currently, imidacloprid is the insecticide with the world's fastest growing sales (Raymond-Delpech et al., 2005, Tomizawa and Casida, 2005). It is marketed under a variety of names, such as Gaucho, Admire, Confidor, Premier, Premise, Provado, Marathon and Winner. Imidacloprid is a broad spectrum systemic neonicotinoid used as foliar treatment, seed dressing or soil treatment in different crops. It is most commonly used in rice, cereal, maize, potatoes, vegetables, sugar beet, peanuts, fruits, cotton. It controls a broad spectrum of pests: aphids, leafhoppers, planthoppers, trips, scales and whiteflies, various species of beetles, leaf miner species, some dipterous pests, termites and locusts. Imidacloprid as a systemic

insecticide, is taken up by plants, primarily through the roots, and transported within the vascular system of the plant to where it can affect plant-feeding pests (Tomlin, 2000). Imidacloprid has contact and ingestion activity. It acts upon the nicotinic receptors and kills insects by either eliciting a neural toxin response with classic toxicity symptoms (i.e., uncoordinated movement and tremors) or by causing a reversible starvation response (i.e., shortened feeding duration, increased test probing, and avoidance) (Nauen, 1995).

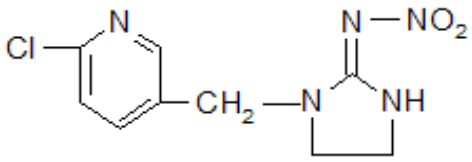

Figure 3: Chemical structure of imidacloprid. (EZ)-1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2- ylideneamine.

Imidacloprid is also toxic to non-target organisms. Imidacloprid is acutely toxic to adult fish at relatively high concentrations. The reported 96 h LC_{50} for common carp (Cyprinus carpio L.) and zebrafish (*Danio rerio* Hamilton) were 280 mg/l and 241 mg/l, respectively (Fossen, 2006, Tišler, 2009). Juvenile fish are considerably more susceptible (Cox, 2001). The LC_{50} for four aquatic organisms including two saline (*Artemia* sp. and *Aedes taeniorhynchus* Wiedemann) and two freshwater (*Daphnia magna* Straus and *Aedes aegypti* L.) organisms has been reported by Song et al. (1997). The LC_{50} values ranged from 0.013 to 361.23 mg/l with *A. taeniorhynchus* being the most susceptible and *Artemia* the most tolerant species.

Imidacloprid is applied as a systemic insecticide to the soil around trees. As such it is likely to cause adverse effects on litter-dwelling earthworms (Kreutzweiser et al., 2007). Capowiez et al. (2005) working on two different species of earthworms *Aporrectodea nocturna* Evans and *Allolobophora chlorotica* Savigny found out that LC_{50} for imidacloprid was between 2 and 4 mg/kg dry soil. In the same study at lower concentrations (0.5 and 1 mg/kg dry soil) weight loss was observed. In the study by Luo (1999) a decrease in enzymatic activity of earthworms was detected when imidacloprid concentration in soil reached 0.1 mg/l. Imidacloprid was shown to be toxic to another group of important decomposers of organic material-terrestrial,

isopods (Drobne et al., 2008). A decrease in weight gain and feeding rate were observed in juveniles of *Porcellio scaber* Latreille, after two weeks of feeding on imidacloprid dosed-food (10 µg imidacloprid/g food). The parameters most affected in adults were the feeding rate and the epithelial thickness of digestive gland.

Imidacloprid was also shown to be toxic to insects that provide an economic benefit to agriculture: pollinators (honeybees, bumblebees), predators (predatory bugs, lady beetles, lacewings) and parasitoids (De Cock et al., 1996, Kunkel et al., 2001, Stapel et al., 2000, Rogers et al., 2007). Suchail et al. (2000) determined the LD_{50} values for *Apis melifera carnica* Pollman: at 24 and 48 h LD_{50} values were 4.5 and 24 ng/bee after oral and contact application, respectively. The LC_{50} values calcualted for the predatory bug *Orius laevigatus* (Fieber) are 0.04 mg a.i./l for nymphs and 2.1 and 0.3 mg a.i./l for adults, via ingestion and residual contact, respectively (Delbeke et al., 1997). Some studies, on the other hand, documented fecundity increase in imidacloprid treated two-spotted spider mite *Tetranychus urticae* Koch (James and Price, 2002).

2.2 Sublethal effects of deltamethrin and imidacloprid on insect behaviour

The behaviour of insects and other animals is governed by interactions among neurons within their nervous system. Ability of most insecticides to kill insects, results from attacking specific sites within the insect's nervous system. Therefore, in addition to direct mortality induced by neurotoxic insecticides, their sublethal effects on insect behaviour and physiology (affecting biochemistry and neurophysiology, development, longevity, immunology, fecundity, sex ratio) have to be considered. A sublethal dose is defined as dose inducing no apparent mortality in the experimental population. The sublethal effects are defined as effects at the physiological or/and behavioural level on individuals that survive exposure to insecticide (Desneux et al., 2007).

Studies of sublethal effects of neurotoxic insecticides on the behavioural patterns are important for several reasons (Haynes, 1988): (1.) detailed observations of

behavioural symptoms of poisoned insects help to elucidate modes of action for novel and conventional insecticides; (2.) insecticides interfere with behavioural patterns of pests, contributing to the management of pest populations; (3.) beneficial insects can be affected by insecticides in a way that is overlooked by screening procedures; (4.) selection may favour insects that specifically respond to insecticides and possibility for development of behavioural resistance could be considered. More than 75% of the ecotoxicological studies from 1950 to 1986 measured mortality of beneficial insects (Desneoux et al., 2007). Median lethal dose, concentration and/or time were measured in another 20% of the studies. Only 5% of the studies reported on sublethal effects, of which mostly reported were reduced fecundity, development and longevity (review in Haynes, 1988).

In recent years, sublethal effects, expressed as modification in behaviour (reproduction, host finding, oviposition, feeding and general locomotory behaviour, dispersal, navigation, orientation, learning performance, communication, etc), have been investigated in an increasing number of studies (review in Desneux et al., 2007). Because of their important value for integrated pest management systems and in pollination processes natural enemies and pollinators, have received most attention. Various sublethal effects of imidacloprid and deltamethrin, two of the most widely used insecticides, were observed in different insects species, again focusing on predators and natural enemies. Some of the examples are described below.

Mobility

Effects of insecticides on mobility have been rarely studied directly; instead, other parameters indicating changes in mobility are reported, such as: knock-down effect, un-coordinative movement, tumbling, abdomen tucking, excessive grooming, self-cleaning behaviour, rubbing the hind legs together, etc. Effects of imidacloprid indicating mobility disruption were reported for different species of bees, parasitoids and predatory beetles (Brunner et al., 2001, Kunkel et al., 2001, Suchail et al., 2001). An increase in stationary behaviour was observed in imidacloprid treated honeybees (Lambin et al., 2001, Medrzycki et al., 2003). Secondary consequences of changed mobility (disruption in detection of kairomones and increased arrestment by

kairomones) were reported for deltamethrin treated parasitoid wasp *Trisolcus basalis* Wollaston (Salerno et al., 2002). Furthermore, deltamethrin was shown to alter mobility and thus distribution in predatory beetle *Coccinella septempunctata* L. due to its repellence and irritant effects (Wiles and Jepson, 1994a). Walking speed of spiders treated with deltamethrin decreased, resulting in their mortality increase, due to increased predation by carabid beetles (Everst et al., 1991).

Orientation

Navigation or orientation can involve multiple sensory cues, such as chemical, visual and vibrational one. Insects spend a significant proportion of their life searching for hosts, prey, food and mates. For pollinators visual learning of landmarks is important in spatial orientation. It enables them to navigate to a food source as well as to find their hive and provides information about the distance and direction of the food to their nest mates (waggle dance). Most of the studies report negative effects of deltamethrin and imidacloprid on orientation behaviour. Treatment with deltamethrin exhibited alteration of the homing flight in honeybees. To return to the hive, treated bees needed three times longer than untreated ones (Vandame et al., 1995). Nectar from imidacloprid-treated cotton reduced the flight response of the parasitoid wasp (*Microplitis croceipes* Cresson) for 4 days, which resulted in reduced host foraging ability (Stapel et al., 2000).

Feeding behaviour

Insecticides may interfere with the feeding behaviour of treated insects in different ways: repellent effect, antifeedant properties of insecticides and disruption in the ability to find food because of reduced olfactory capacity (Decourtye and Pham-Delegue, 2002). After exposure to deltamethrin or imidacloprid, honeybees exhibited lower syrup consumption and decreased foraging activity (Decourtye et al., 2004b, Ramirez-Romero et al., 2005). Pyrethroids are probably the best known repellent insecticides (Rieth and Levin, 1988). Because of repellence honeybees consume less of the substance, hence deltamethrin compared to imidacloprid exposure, may result in less deleterious effect on bees. However, if the effect lasts too long after the

exposure, the bees may die from starvation. The opposing effects of attraction to host-derived kairomones and repellence from deltamethrin were also reported for aphid parasitoids (Hymenoptera: Aphidiinae) (Longely and Jepson, 1996). Parasitoids responded strongly to patches of aphid honeydew on a filter paper, but adding deltamethrin caused early departures from the feeding areas because of the repellence or/and irritant effect. Furthermore, the impact on food detection may also have negative impact on feeding capacities of parasitoids. The influence of deltamethrin on general feeding behaviour was examined in the predatory ladybeetle *Coccinella septempunctata* (Wiles and Jepson, 1994a). The movement of ladybeetles on deltamethrin treated plants increased; they tended to stay on plant parts that received lower doses of the insecticide, which indicates repellent effect of deltamethrin at higher doses. An antifeedant effect was observed for the predatory carabid *Nebria brevicollis* Fabricius feeding on deltamethrin treated aphids; majority of beetles, ingesting treated aphids, exhibited a regurgitation response after consumption (Wiles and Jepson, 1993).

Learning performance

Effects of insecticides on the learning process (important for foraging) have been studied on honeybees. Under laboratory conditions, olfactory learning ability can be studied using bioassay based on conditioning of proboscis extension response (PER) (Decourtye and Pham-Delegue, 2002, Decourtye et al., 2005). Using a conditioned PER response assay, honeybees can be trained to associate an odour stimulus with a sucrose reward. By this approach authors observed reduced learning performance in bees after chronic exposure to imidacloprid or deltamethrin (Decourtye et al., 2003, Decourtye et al., 2005). The PER bioassay can also be used for investigation of effects of insecticides on different parameters of memory during olfactory conditioning of PER. When evaluating the effect of imidacloprid on different memory processes, the impairment of medium-term memory was observed, while they observed no difference in short- and long-term memories (Decourtye et al., 2004a).

Oviposition behaviour

Most studies concerning the effects of insecticides on oviposition behaviour have been conducted on parasitoids. Oviposition and parasitism rate of parasitoids affect the efficiency of pest control. Insecticides can disrupt the coordination between the insect nervous and hormonal systems, resulting in a breakdown within the complex of behavioural and physiological events related to oviposition. Changes in oviposition can be related to repellent effect of the insecticide and the insects will avoid the treated sites, i.e. hosts as suitable oviposition sites (Longely and Jepson, 1996). On imidacloprid treated leaves host searching efficiency of parasitoid wasp (*Neochrysocharis Formosa* Westwood) was reduced; on imidacloprid treated leaves, the wasp encountered, oviposited and fed on hosts less frequently (Tran et al., 2004). On the other hand, in another species of parasitoid wasp (*Tiphia vernalis* Rohwer) no change in parasitism efficiency was observed after imidacloprid exposure (Oliver et al., 2005). Reduction in oviposition could be a consequence of some other effects, such as modification of mate-locating, courtship behaviour and mate communication.

Communication

Most general definition of communication involves the provision of information by a sender to a receiver (Bradbury and Vehrencamp 1998). The sender emits a signal, which conveys information. The signal is transmitted through the environment and is detected by the receiver. The receiver uses the information as help to make a decision about how it should respond. The receiver's response affects the fitness of the sender as well as its own. Communication plays a central role in animal social and/or solitary species. Animals convey different types of messages to each other: information about their identity (sex, group and individual identity), status and mood (e.g. dominancy, aggressiveness), intention of activity, relevant discoveries (e.g. communicating about predators and prey presence, food source), etc. There are interfaces between communication and many areas of biology, including evolution, population genetics, physiology and neurobiology. Results obtained by research of

investigating communication are applied in pest control, conservation and animal welfare.

The environment in which communication takes place, as well as the physiological and morphological adaptations that both sender and receiver use to do the task, may severely limits the properties of the signal forms. The most common signal modalities are hearing, vision, smell, taste and vibration detection sense, sense of touch and electrical sense. Signals are conveyed by different transmission media such as air, water, sand, soil, plants, etc.

2.3 Sublethal effects of insecticides on communication

Very low number of studies investigated effects of insecticides on communication and has been focused mainly on disruption of chemical communication between sexual partners and disruption in the detection of kairomones of host species in the case of parasitoids. Neurotoxic insecticides modify chemical communication by altering the capacity for stimulus creation by the emitter or stimulus perception by the receiver. Deltamethrin treated parasitoid wasp males (*Trisolcus brassicae*) exhibited increased arrestment by female sexual pheromone, whereas when females were treated, their pheromones were less arresting for males (Delpuech et al., 1999). Furthermore, after deltamethrin exposure the wasp responded less to its host kairomone (Salerno et al., 2002). Sublethal doses of deltamethrin significantly affected the chemical communication in the Asian corn borer as well (Yang and Du, 2003); after the treatment they observed a decrease in the calling behaviour and pheromone production, males orient toward and locate the female less frequently. Sublethal exposure to pyrethroids decreases the duration of calling behaviour of the pink bollworm moth females (P*ectinophora gossypiella* Saunders), male response to the sex pheromone and alter close range mating behaviour in male codling moth (*Cydia pomonella* L.) (Haynes and Baker, 1985, Moore, 1988, Krupke et al., 2002).

However, to date no studies have examined the effects of sublethal doses of neurotoxic insecticide on substrate-borne communication.

2.4 Substrate-borne communication

Communication with vibrational signals transmitted through the substrate (i.e. substrate-borne communication) provides the information used in predator-prey interactions, recruitment to food source, mate choice, intra-sexual competition and maternal-brood social interactions for many species from arthropods to vertebrates (Hill, 2001). In the following text the term vibrational communication and vibrations are restricted to substrate-borne signalling.

Only in the last three decades, the awareness about vibrational signals as biologically relevant information has increased (Hill, 2001). Vibrational communication represents a primitive form of signalling from which airborne auditory communication has evolved (Stumpner and von Helversen, 2001, Stritih and Stumpner, 2009). Compared with vertebrates, the mechanism of production, detection and response to vibrations, is much more investigated and known in arthropods. Spiders use vibrational information for prey-catching, territorial behaviour and courtship behaviour (Barth, 2002, Elias et al., 2003). In spider *Cupineius salei (*Keyserling*)* species specificity of the male vibrational signals serves the female to recognize the conspecifics (Schuch and Barth, 1990). During their courtship behaviour, males of the sand fiddler crab (*Uca pugilator* Bosc), drum on the ground with their chelae and produce vibrational signals (Aicher and Tautz, 1990). Scorpion, *Paruroctonus mesaensis* (Stahnke), uses vibrations to detect direction and distance of prey in sand (Brownell and Farley, 1979).

In insects, vibrational communication is far more prevalent than airborne sound (Cocroft and Rodríguez, 2005). Communication with different mechanical signals has been identified in 92% of identified insect species and 71% of them exclusively use vibrational signalling. With development of more precise methods for recording and analysing of the substrate-borne signals, investigation of diversity of insect vibrational communication has been intensified in recent decade. Vibrational communication has been found in most of the insect groups: Ortophtera, Isoptera, Plecoptera, Psocoptera, Stenorryncha, Auchenorryncha, Heteroptera, Megaloptera, Raphidoptera, Neuroptera, Coleoptera, Mecoptera, Diptera, Trichoptera,

Lepidoptera, Hymenoptera, Thysanoptera, Zoraptera, Embiidina, Blattodea (Cocroft and Rodriguez, 2005). Vibrational signals are important in a variety of interactions and have several functions. In ants, beetles, group-living treehoppers and Heteroptera vibrations are involved in defensive behaviour (Cocroft, 1999, Cocroft et al., 2000, Fuchs, 1976, Serrano et al., 2003, Gogala, 1985). Some insects use vibrations in predator-prey interactions. Caterpillars and butterfly pupae produce vibrational signals for protection against predators and evaluation of predation risk (DeVries, 1990, Castellanos and Barbosa, 2006). Vibrations serve termites in pathogen and predator alarm behaviour (Rosengaus et al., 1999, Reinhard and Clément, 2002). Predatory pit-building antlion (*Euroleon nostras* Fourcroy), hemipteran predators and egg parasitoids use substrate vibrations for detecting and locating their prey (Pfannenstiel et al., 1995, Laumann et al., 2007, Mencinger-Vračko and Devetak, 2007). Vibrational communication is also important in a variety of other behavioural contexts (locating and remaining in a group of conspecifics, locating food resource) and is crucial for the success of group-living, herbivorous insects (Cocroft, 2001). However, most of the investigated vibrational signals in different insect groups are associated with sexual behaviour, to attracting and finding a partner (Field and Bailey, 1997, Kanmiya and Sonobe, 2002, Stewart and Sandberg, 2006, Claridge, 1985, Cocroft and McNett, 2006, Virant-Doberlet and Žežlina, 2007, Mazzoni et al., 2008, Devetak, 1998, Goulson et al., 1994, Kasper and Hirschberger, 2005, Čokl and Virant-Doberlet, 2003, Kanmiya, 2006).

The Heteroptera or "true bugs" is a suborder of the insect order Hemiptera. It is estimated that Heteroptera contain some 37.000 described species, and perhaps 25.000 are yet to be described (Schaefer and Panizzi, 2000). Heteroptera contains eight infraorders among which representatives of Cimicomorpha and Pentatomorpha represent those with most investigated examples of vibrational communication (Gogala, 2006). The family Pentatomidae (stink bugs) is the third largest family within Heteroptera (Panizzi et al., 2000). It represents a large group of phytophagus (many of them economically important pests) and predator species (McPherson and McPherson, 2000a). Inter-sexual and intra-specific communication in many stink bug species is mediated by both chemical (Millar, 2005) and vibrational signals (Čokl, 2008).

2.5 Nezara viridula

2.5.1. Biology

N. viridula is one of the economically most important and widespread Heteroptera species in the world, occurring through-out the tropical and subtropical regions of Europe, Asia, Australia, Africa and Americas (Todd, 1989, Panizzi et al., 2000). It is proposed that the area of origin of *N. viridula* was the Ethiopian region (Hokkanen, 1986, Jones, 1988). The species is highly invasive; newly established populations have recently been reported in Hungary (Redei and Torma, 2003), the Galapagos island (Henry and Wilson, 2004) and England (Barclay, 2004). There is a growing concern that due to its high vagility (Jones, 1988), polyphagous feeding habits and its ability to move to alternative hosts (Panizzi, 1997, Panizzi et al., 2000), in combination with global warming (Musolin and Numata, 2003) and the changes in agricultural practices, *N. viridula*, as well as other stink bugs, will soon become even more wide spread and will represent even greater economical problem in agriculture.

The species feeds on cultivated and uncultivated plants of more than 30 families of dicotyledonous and monocotyledons (Todd, 1989, Panizzi 1997, Panizzi et al., 2000). It is one of the most important stink bug pests of soybean in the world. It is also an important pest of legumes and brassicas (Panizzi, 1997), rice, corn, tobacco and a number of vegetable crops, such as tomato, sweet pepper, eggplant (McPherson and McPherson, 2000b) and also macadamia (Jones et al., 2001), pecan nuts (Coombs, 2000). In recent years, it has re-emerged as a significant pest of cotton (Greene et al., 1999, Willrich et al., 2004).

Barrel-shaped, white eggs are laid in clusters, closely packed in regular rows, firmly glued to each other and to the substrate (Todd, 1989). An egg mass usually consists of 80 to 120 eggs, which usually hatch in four to nine days. As eggs mature they become deep yellow, then pinkish-yellow, and finally bright orange at eclosion. Eggs are deposited in the upper regions of crops on the under-surface of leaves or pods. Egg hatching is temperature dependent and the synchronization of hatching of an egg mass is determined by the emergence of nymphs, which stimulate hatching in

neighbouring eggs (Lockwood and Story, 1986). Twenty-four to 60 days after hatching *N. viridula* moults five times before reaching maturity. Duration of development of nymphal stages depends on temperature and on host food supplies. First instars cluster on or near the egg mass apparently do not feed (Velasco and Walter, 1992). Adults live for up to three weeks in hot summer weather and longer over winter. *N. viridula* usually appears in 2 generations per year, but in favourable environmental conditions and geographical areas they detected up to five generations per year (e.g. in south Florida) (Todd, 1989, Velasco et al., 1995). In regions with cold winter the autumn generation lives longer, overwintering or diapausing in debris, under bark or in buildings (Todd, 1989). While most diapausing *N. viridula* turn into a reddish brown colour, this is not the case for all individuals (Seymour and Bowman, 1995). Diapause is controlled by a long-day photoperiodic response with the critical day-length for diapause falling into a narrow range close to 12.5 h (Musolin and Numata, 2004). Although reproduction ceases during overwintering, survival is improved by feeding and adults often feed during mild periods on mustard (Brassica spp.) and wild radish (*Raphanus* spp.) (Todd, 1989). As temperature increases in spring, adults leave overwintering sites and begin to feed and mate in clover, small grains, early spring vegetables, corn, tobacco and weed hosts (Todd, 1989). Preference for particular host plants changes with host maturity and phenology (Todd and Herzog, 1980, McPherson and McPherson, 2000b). Host plants are most attractive during fruit formation, as fruits mature, stink bugs move to more succulent plants. In temperate regions midsummer hosts include tomatoes, leguminous weeds, vegetables, row crops, cruciferous vegetation and okra. In late summer and early fall soybean is the major source of food, for the third generation.

Adults and nymphs attack stems, foliage (particularly leaf veins), flowers and fruits, but prefer young, tender growth and fruiting structures (Todd, 1989). This bug is primarily pod seed and fruit feeder, so its population peak coincides with or lags slightly behind the development of fruiting stages of the primary host species (Panizzi et al., 2000, McPherson and McPherson, 2000b). First step in the incidence of the host plant damage is mechanical injury; the bugs pierce the seed or fruit to feed on plant juices. Second step is chemical injury, caused mainly by degradation of cells with digestive enzymes. Finally, hormonal and physiological imbalances cause

fruit and tissue malformation, abscission of reproductive organs and altered vegetative growth. Significant reduction in yield, quality and germination can result from feeding by *N. viridula*. Furthermore, mechanical damage done by feeding also provides entrance points for pathogenic organisms, which then cause additional damage.

Several tactics have been studied and implemented for controlling populations of *N. viridula* (Knight et al., 2007). Controlling measures mainly based on the use of conventional insecticides, including a number of carbamates, organophosphates, some pyrethroids (Jackai et al., 1990, Greene et al., 2001, Panizzi et al., 2000) and insect growth regulators (Riba et al., 2003). The use of natural insecticides, such as extract of neem seed, also decreased the damage caused by *N. viridula* (by disruption of feeding behaviour and increased mortality) (Seymour et al., 1995, Durmusoglu et al., 2003, Singha et al., 2007). Several efforts have been made to use other tactics within the context of agricultural practices and biological control to suppress outbreaks of the southern green stinkbug. Trap cropping has been utilized to suppress *N. viridula* on soybean (Panizzi et al., 2000). *N. viridula* is attacked by numerous natural enemies, including predators, parasitoids and entomopathogenes. Jones (1988) has compiled an extensive list of *N. viridula* parasitoids worldwide. The most important one are the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) and parasitoids of the genus *Trichopoda* (Tachinidae: Diptera), which attack large nymphs and adults of *N. viridula* (Todd, 1989, Corrêa-Ferreira and Moscardi, 1996, Panizzi et al., 2000). Several arthropod predators (e.g. grasshoppers, fire ants, spiders, predatory stink bugs) feed on *N. viridula* and predation is also recognized to be an important mortality factor for *N. viridula* (review in Todd, 1989, McPherson and McPherson, 2000b, De Clercq, et al., 2002). Several biotic and abiotic factors modulate the abundance of *N. viridula* populations (Todd, 1989). Eggs are destroyed mainly by parasites and predators; first instars by excessive rainfall, low humidity and high temperature; second instars by predators, such as spiders and ants; later instars and adults due to moulting difficulties and finally adults by parasitoids.

2.5.2. Mating behaviour and vibrational signals

The southern green stink bug has a complex mating behaviour implicating chemical and vibrational signals. Mature males produce species specific sex pheromone that acts as long range attractant for mates (Aldrich et al., 1987, Borges et al., 1987, Brézot et al., 1994). When reaching the same plant *N. viridula* communicate predominantly with vibrational signals transmitted through the substrate (Čokl and Bogataj, 1982, Ryan et al., 1996). During mating behaviour males and females produce four different species and sex specific signals called songs (Čokl et al. 2000, Figure 4). The female calling song (FCS) is usually the first one emitted and was found to be the most important for mate localization (Čokl et al., 1999, Ota and Čokl, 1991). Female calling song triggers males to respond with the calling (MCS) and/or courtship song (MCrS), which keep female singing with a steady signal repetition time (Čokl and Bogataj, 1982). Furthermore, FCS activates the male to walk and triggers its characteristic searching behaviour, which includes stopping at stem junctions, where the male straddles his legs and antennae across the junctions and waiting for the next female signal (Ota and Čokl, 1991). Female calling song increases both the percentage of males that release the pheromone and the amount released per male (Miklas et al., 2003b). Females also produce the courtship song (FCrS) of which temporal characteristic resemble male courtship song, but has a specific frequency characteristic. Along with chemical and vibrational signals, other signal modalities, such as vision, touch and near-field airborne sound signals are used during mating behaviour (Čokl, et al. 2000).

Each song of *N. viridula* vibrational repertoire is characterized by specific frequency and temporal characteristics, but little is known about their species specificity and conspecific song recognition. All stink bug species that have been investigated to date show no species specific frequency characteristic. They efficiently communicate through their herbaceous host plants with narrow-band low frequency signals (Čokl et al., 2000). The fundamental frequencies range between 70 and 180 Hz and their harmonics do not exceed 500 Hz (review in Čokl, 2008). Moreover, recent studies show that frequency characteristics of songs and one of the subgenual receptor cells (Čokl, 1983) of the southern green stink bug are tuned to the resonant properties of

their herbaceous host plants (Čokl et al., 2005). On the other hand, some studies already suggested that mate choice is based on the temporal characteristics of vibrational signals and specifically in *N. viridula* species specificity of FCS (Miklas et al., 2001, Miklas et al., 2003a, Hrabar et al., 2004). Males of a specific population recognize and respond to FCS of other populations, but they show a clear preference for the song of their own population (Miklas et al., 2003a). *N. viridula* males can distinguish between conspecific and heterospecific songs, but only if they differ in temporal characteristics (Hrabar et al., 2004). When males were stimulated by the female call of *Acrostermum hillare* (of temporal characteristics different from FCS), their vibrational and locomotory behaviour was inhibited (Miklas et al, 2003a). In contrast, in an early study of Čokl and co-workers (1978) low species specificity was exhibited by males; vibrational signalling of males were triggered by conspecific and heterospecific female calling song of sympatric species *Palomena prasina* (of different temporal characteristics) and even human voice imitating FCS.

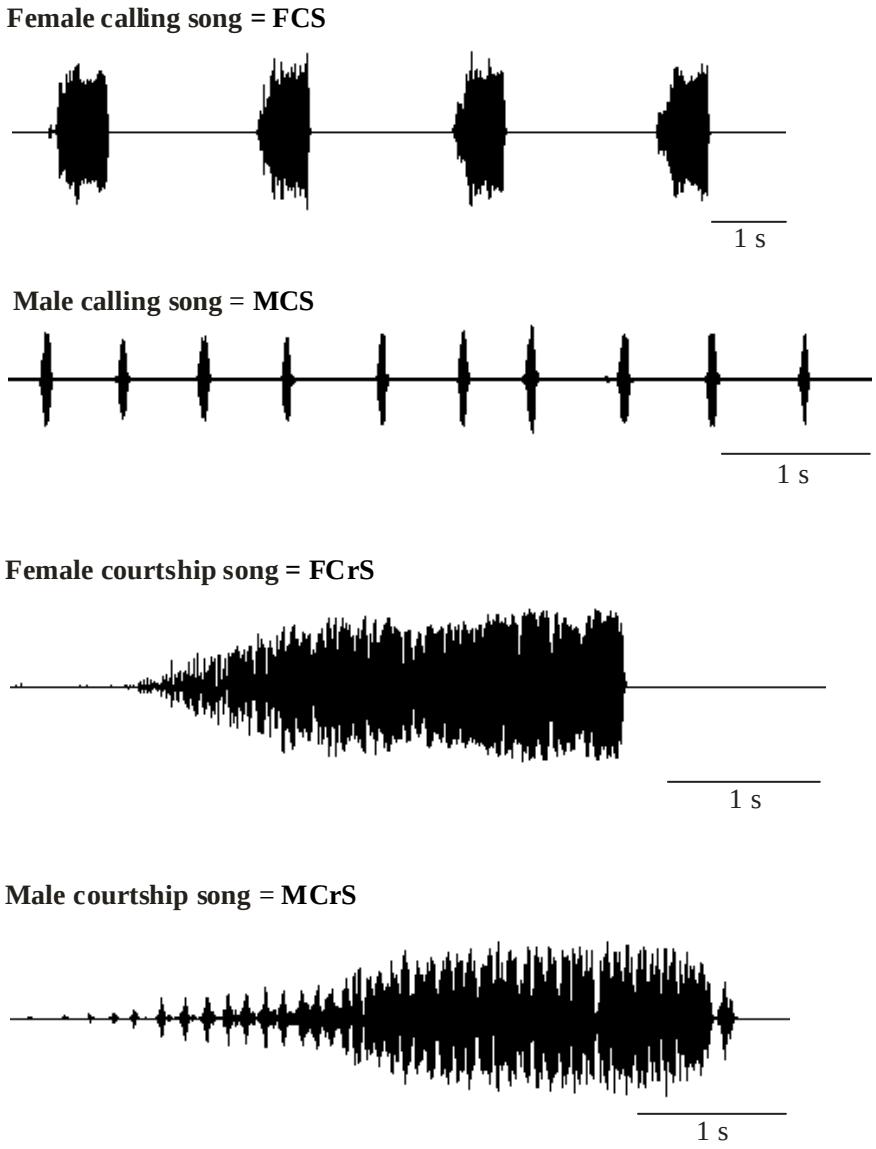

Figure 4: Oscilograms of female and male vibrational signals of the southern green stink bug, *Nezara viridula*.

2.5.3. Mechanism of signal production

The first and second abdominal tergites of stink bugs are fused into forward-backward movable plate. The plate is loosely fixed anteriorly to the thorax and posteriorly to the third abdominal tergum by a chitinous membrane, and more firmly laterally to the pleurites (Kuštor, 1989). In freely moving and singing animals the tergal plate longitudinal and lateral compressor muscles contract simultaneously and cause vertical movements of the abdomen (Amon, 1990). The first and the second of

tergal longitudinal muscles move the plate forward and backward and contraction of compressor muscles moves it in a latero-ventral direction. Multiple peaks of electromyogram potentials indicate that motor units within a muscle are not coupled, but contract one after another with a short time delays. Repeated muscle contractions vibrate the abdomen in the dorso-ventral direction and the vibrations are transmitted through the legs to the substrate. The velocity of signals recorded from the body of a singing bug ranged between 0.1 and 1.0 mm/s (Čokl et al., 2007).

2.5.4. Vibrational receptors and information processing

In *N. viridula* receptors detecting vibrations are located in and on all six legs: the subgenual organ inside the tibial hemolymph channel, and joint chordotonal organs and nongrouped campaniform sensila (Michel et al., 1983, Čokl et al., 2008). Antennae are used as additional mechanosensory input during searching behaviour (Ota and Čokl, 1991) and during mate antennation (Kon et al., 1988).

The subgenual organ is composed of two scolopidia, each of them with one sensory cell. The two cap cells form the flat ligament, which is distally fixed to the cuticle and bends freely in the hemolymph. The sensory nerve with axons of both sensory cells joins the nerve innervating the femoral chordotonal organ. Each leg is equipped with three chordotonal organs: the femoral chordotonal organ (composed of 12 scolopidia), the tibial distal chordotonal organ (two scolopidia) and the tarso-pretarsal chordotonal organ (two scoloparia). Receptor cells of all sensory organs terminate in the ipsilateral side of the protoracic ganglion (for the front legs), and at the meso- and meta-toracic part of the central ganglion (for the middle and hind legs). Different vibrational receptor neurons were identified and divided into low- and high-frequency detectors (Čokl, 1983). The low-frequency receptor neurons (LFR) (attributed to the chordotonal organs and/or the campaniform sensila) respond optimally to stimuli below 120 Hz frequency in a phase-locked manner, with threshold velocity between 0.03 and 0.06 mm/s. The higher frequency receptor neurons (attributed to the subgenual organ) are of two types: middle frequency receptor neurons (MFR) and higher frequency receptor neurons (HFR). The MFR are

tuned to frequencies around 200 Hz (velocity threshold above 0.01 mm/s) and the HFR to frequencies between 750 and 1000 Hz (velocity threshold around 0.002 mm/s).

Campaniform sensila, Johnsoton's organ and the central organ were described in and on the antennae (Jeram and Čokl 1996). Twelve campaniform sensila were identified on the surface of antenna. The Johnston's organ is located in the distal part of the third antennal segment and is composed of 45 amphinematic scolopidia. The central organ, composed of seven mononematic scolopidia, is located in the pedicel. The best sensitivity of both organs was determined at 50 Hz with a threshold around 2 mm/s; below 100 Hz, the responses are phase-locked (Jeram and Čokl, 1996). Receptor cells terminate in the lateral deutocerebrum, subesophageal ganglion and in the central ganglion, with some of the projecting into its abdominal neuromeres.

Central processing of the vibrational information detected by leg vibrational receptors of *N. viridula* was first investigated by Čokl (1983). Four vibrational interneuron types were identified and classified (in the thoracic part of the ventral cord (Čokl and Amon, 1980). Interneurons showed different frequency-intensity characteristics: between 100 and 600 Hz and between 600 and 1500 Hz. In addition, Zorović and co-workers (2008) described morphology and function of 10 different types of higher order vibrational neurons in the thoracic ganglia. Based on their frequency sensitivity interneurons were divided into two groups: the low frequency units, which are tuned to 50 Hz, and the middle frequency units tuned to 200 Hz.

2.5.5. Transmission of the vibrational signals

Communication signals have to incorporate, among others, the information of the identity and location of the sender. It is essential for communication that signals travel successfully over behaviourally relevant distance while retaining the original information and characteristics. Insects producing air-borne acoustic signals have to be relatively large in respect to the wavelength of emitted signals (efficient radiation of a sound pressure wave is possible only in a frequency range for which the

radiator's diameter is above 1/3 of the radiated wavelength) (Markl, 1983). Herbaceous plants are the common environment of most insects and most of them exchange signals through the plant tissue (Cocroft and Rodriguez, 2005). The major reason is their small body size, which prevents efficient emission of low frequency airborne signals in thick vegetation (Markl, 1983). In their pioneering study Michelsen and co-workers (1982) demonstrated among others that insects use dispersive (i.e. the propagation velocity of waves is frequency dependent) bending waves for communication. Furthermore, standing wave conditions probably occur in rod-like structures such as stems and stalks because of reflections and low attenuation of the transmitted signals. Recent study showed that non-dispersive bending waves can be expected in stems of larger diameter but at frequencies above 4 kHz (Casas et al., 2007). Cocroft and co-workers (2006) described the influence of physical properties of different plant substrates on insect communication.

All stink bugs, investigated so far, emit spectrally similar signals with narrow frequency peaks, in most cases below 600 Hz, with fundamental frequency around 100 Hz (for review see Čokl, 2008). Propagation of low frequency vibrational signals depends on characteristics of the signal and on the mechanical properties of the stem. These signals propagate through herbaceous plants with very little attenuation (low-pass filtering) (Michelsen *et al*. 1982, Barth 2002, Cocroft et al. 2006, McNett and Cocroft, 2008), therefore they are well suited for long distance communication. Čokl and co-workers (2007) studied transmission of communication signals of *N. viridula* and *Murgantia histrionica* (Hahn) through different plant substrates: *Cyperus alternifolius* L., *Phaseolus vulgaris* L., *Hedera helix* L., *Isomeris arborea* Nuttall, *Sysimbrium irio* L. The signals were attenuated or amplified during transmission from insect body to plant surface in relation to mechanical properties of the plant. They recorded up to 6 dB attenuation or amplification of the signal by transmission from the body to the plant surface and up to 20 dB attenuation by transmission from the leaf to the stem. Attenuation rate during transmission through the green parts was lower than in woody part of the plant. Signal velocity decreases non-linearly with the distance; regularly repeated velocity minima (nodes) and maxima (antinodes) were observed in herbaceous but not in woody plants.

3 EXPERIMENTAL METHODS

3.1 Experimental animals

Sexually mature adult of the southern green stink bug, *N. viridula,* used in this study, were collected in autumn at the North Adriatic coast, Slovenia. The adult bugs were divided according to sex and maintained in the laboratory under diapause conditions (in darkness at 5–10°C) for one to three months. The diapausing males were afterwards transferred to rearing conditions: 22°C±1SD, 70–80% relative humidity, with a photoperiod of L: D being 16:8 h (lights on at 08:00 and off at 24:00 hours). Each individual was placed in a separate plastic cup (14 cm deep, 6 to 10 cm diameter, bottom to top) and reared on a diet of green bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.) plants and roasted peanuts (*Arachis hypogaea* L.). To ensure that the males were sexually active and responsive to female signals, they were used in experiments only after ten days (or more) under rearing conditions.

3.2 Playback experiment

Playback experiments were conducted in an anechoic and sound insulated chamber (Amplifon, Fa. Amplaid, Italy) at room temperature (21–23°C) and humidity (50–65%). Males were stimulated by the natural female calling song (FCS), pre-recorded from the loudspeaker membrane and by artificially synthesised FCS (described below). Vibrational stimuli were applied directly from a computer to the low-middle frequency loudspeaker membrane (Conrad Electronic, impedance 8W, 4.5 mm diameter, 50–2000 Hz). The test male was placed on the loudspeaker. The level of the stimulation output velocity was adjusted to the level of the tested male response velocity (0.1–0.2 mm/s). Velocity is defined as a vector quantity that specifies time rate of change of displacement. Male vibrational responses (i.e male calling song (MCS) and courtship song (MCrS)) (Figure 4) were recorded from the membrane surface with a laser vibrometer (controller Type 2200-L, sensor head Type OFV-353,

Polytech GmbH, Waldbronn, Germany) and stored in a computer with Cool Edit Pro (Syntrillium Software, Phoenix, USA) software at the sampling rate of 48000 Hz. Recordings were analysed using the computer programs Sound Forge 6.0 (Sonic Foundry 2002) or Raven 1.2. (Charif et al., 2004). All experiments were conducted on a non-resonant loudspeaker membrane, which reproduces natural and artificial stimuli without affecting their temporal and spectral properties (Čokl et al., 2005).

Stimulation program included two sequences (synthesised and natural FCS) each composed of 35 pulse trains separated by 3 min no-stimulation period (Figure 5). Pulse was defined as unitary homogenous parcel of vibrations of finite duration (Broughton 1963). Pulses arranged into repeatable and temporally distinct groups were termed a pulse train. The artificial FCS was synthesised by the use of Sound Forge (Sonic Foundry, Inc. Madison, USA) software. The characteristics of the synthesised song resembled mean values of parameters determined for the natural FCS of 5 females of the Slovene population (Table 2). Male responses to synthesised FCS did not differ significantly from responses to the natural FCS, therefore the synthesised FCS was used in the subsequent preference tests in which the parameter values had to be varied (Table 3). With synthesised FCS it was possible to obtain and control the stimulus characteristics defined by specific parameter values. The sequence of natural FCS was played to the males after the synthesised FCS sequence and was daily used to monitor the general responsiveness of tested males.

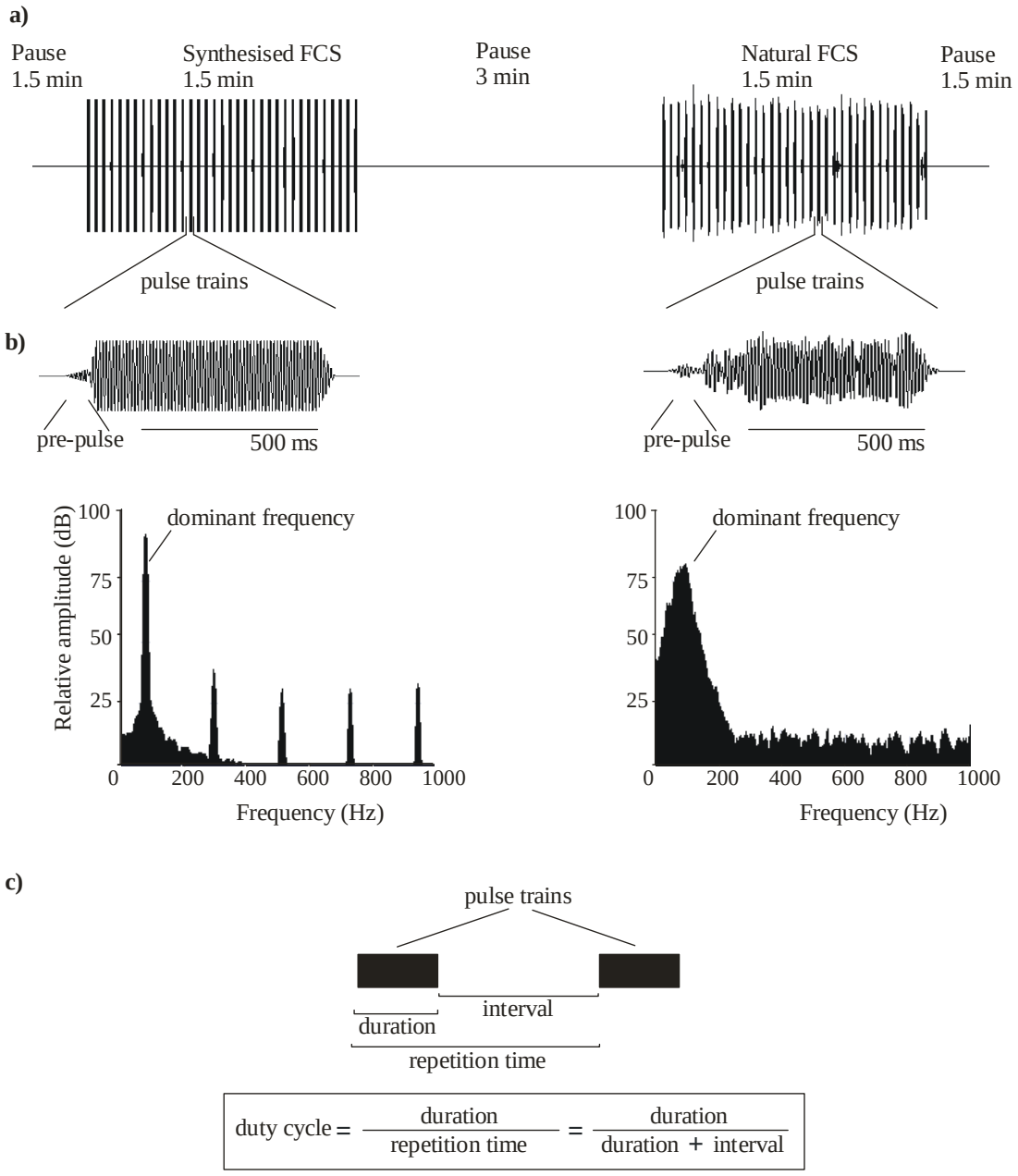

Figure 5: Characteristics of the *Nezara viridula* female calling song (FCS) stimulation program. (a) Stimulation program used in the playback experiments (schematic drawing) consisted of pause sections and sequences of 35 pulse trains of synthesised FCS, followed by the natural FCS. (b) One pulse train of synthesised FCS with spectrogram (left); one pulse train of the natural FCS with spectrogram (right). (c) Schematic diagram of the FCS temporal parameters: pulse train duration (=duration), inter-pulse train interval (=interval), repetition time and duty cycle.

Table 2: Parameter values of the natural female calling song (FCS) and the synthesised FCS (mean±SD). Five females were tested (N=5) and 35 pulse trains for each female were measured (n=35).

	Natural FCS (mean±SD)	Synthesised FCS (reference value)
Pre-pulse duration (ms)	80±35	80
Pre-pulse dominant frequency (Hz)	134±15	134
Pulse train duration (ms)	720±37	700
Inter-pulse train interval (ms)	1912±93	1900
Pulse train dominant frequency (Hz)	105±6	105

Table 3: Male responses (male courtship song, MCrS) to the synthesised and the natural female calling song (FCS) sequence. The mean values (±SD) of MCrS parameters were calculated for each male. The response values were compared between males stimulated with the synthesised and the natural FCS (Steel test). Twenty males were tested for each stimulation.

	Number of MCrS	Duration of MCrS (ms)	Response latency 1 (number of FCS)	Response latency 2 (ms)**
Response to synthesised FCS sequence	11.4±6.1	1495±268	8.1±3.5	993±95
Response to natural FCS sequence	11.8±6.9	1639±435	7.7±6.1	989±142
Response to synthesised vs. natural FCS	P=0.837 NS	P=0.382 NS	P=0.821 NS	P=0.909 NS

* Response latency 1=number of the FCS pulse trains needed to elicit the male response.
** Response latency 2 (ms)=time lap between onset of the FCS and onset of male response to the specific FCS (measured in ms)

3.3 Experiment 1-Male preferences for female calling song (FCS) parameters

Pulse train duration, inter-pulse train interval (=interval), duty cycle, repetition time and dominant frequency of FCS

Male preferences for different temporal (Figure 5c) and spectral characteristics were tested with artificially synthesised FCS in which one of the parameters varied while others were held constant, at the mean values of the natural FCS (Figure 6). Duration of the pulse train varied between 200 and 9000 ms (Figure 6a), the inter-pulse train interval (interval) varied between 300 and 10000 ms (Figure 6b) and the dominant frequency between 50 and 1000 Hz (Figure 6d). As a consequence of independently varied pulse train and interval duration, the repetition time and duty cycle varied as

well. Duty cycle is defined as the proportion of time an individual spent singing (Mason, 1996, Fullard, 2006) (i.e. percentage of the time calling occupied by vibration: duration of pulse train/repetition time). To test the importance of duty cycle, relative to the absolute values of pulse trains duration and interval, males were stimulated with the synthesised FCS the duty cycle of which was held constant at 27% (characteristic of the natural FCS) by simultaneously varying the pulse train duration and interval values (Figure 6c). The repetition time was defined as the time between the onsets of two consecutive pulse trains and is therefore a composite parameter (i.e. duration of pulse train plus interval between consecutive pulse trains). In order to evaluate the relevance of repetition time for FCS recognition we conducted 3 additional tests in which the repetition time was kept constant (at the value characteristic of the natural FCS, 2600 ms) by simultaneously varying the pulse train duration and interval outside the rang of the natural FCS (200 ms (duration) and 2400 ms interval), 1500 and 1100 ms, 2000 and 600 ms, Figure 6e). The number of males tested in each set of tests varied between 14 and 26. The order, in which the males were tested, and the order of the stimulation program, used in a particular test, were randomized. Each bug was tested only once per day and with only one type of synthesised FCS stimulus. The day before preference tests, each male was exposed to the control stimulation. Males who were not responding to the control stimuli were eliminated from further experiments.

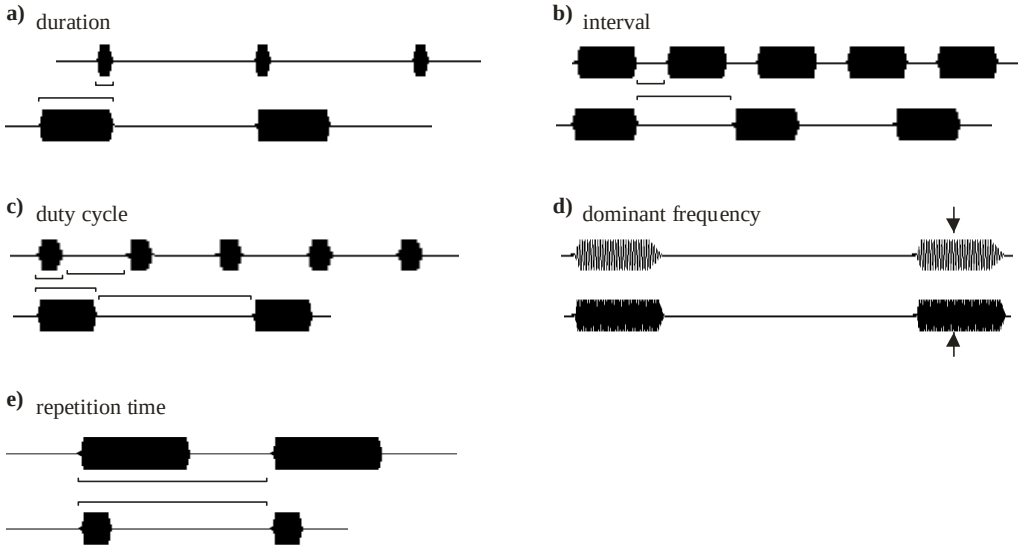

Figure 6: Schematic drawing of stimulation used in the male preference tests. a) Variable pulse train duration with constant interval and dominant frequency. b) Variable interval with constant pulse train duration and dominant frequency. c) Constant duty cycle with variable pulse train duration and interval. d) Variable dominant frequency with constant pulse train duration and interval. e) Constant repetition time with variable pulse train duration and interval.

Pre-pulse of the FCS

In the second set of experiments we investigated the relevance the FCS pre-pulse (Figure 5b) frequency and velocity characteristics for FCS recognition. In the synthesised FCS sequence the frequency of the pre-pulse varied from 70 to 250 Hz and velocity from 0 to 0.816 mm/s, while duration of the pre-pulse, and the characteristics of the main part of the pulse train, were held constant at the mean value of the natural FCS.

Female courtship song (FCrS)

Along with the calling song, females of *N. viridula* also produce the female courtship song (FCrS, Figure4) with pulse train duration of 3900±1179 ms (nine females and 135 pulse trains tested) and dominant frequency similar to that of the male courtship song and without regular interval (Čokl et al., 2000). To examine the response of males to the FCrS, experiments were carried out as described above (for tests of responses to FCS) except that the stimulation programme consisted of a sequence of

35 pulse trains of pre-recorded natural FCrS sequence (from 5 females of Slovene population) followed by natural FCS sequence. In this set of experiments we tested and analysed responses of 21 males.

Data analysis

We calculated the percentage of males responding to each stimulation song (dichotomous scoring of response: response/no response) and present the results as the general responsiveness of males. Male responsiveness to stimulus of a particular stimulation characteristics was compared to the responsiveness to the stimulus of reference value (i.e. value characteristic of the natural FCS: duration 700 ms, interval 1900 ms, dominant frequency 105 Hz) using two-tailed Fisher's exact test. We further analysed the activity of vibrational signalling of males that responded to stimulation (separately MCrS and MCS signalling activity). Signalling activity was defined as the total duration of the response during stimulation (measured as the sum of durations of all emitted MCrS or MCS) and expressed as the percentage of duration of the specific stimulation (i.e. the sequence of 35 synthesised FCS). Kruskal-Wallis test with Steel post hoc test were used to compare signalling activity of males during each of stimulation programs to that of the reference stimuli with characteristics of the natural FCS. Since pulses of MCS are much shorter than those of MCrS (Čokl et al, 2000), the calculated MCS signalling activity was generally lower than the one of MCrS. The effective range of the parameter was defined as the range of parameter values which elicited the same or not significantly lower response as the reference stimulation. Statistical tests were conducted using Kyplot software.

3.4 Chemicals

In the study we used formulated insecticide imidacloprid (Confidor® 200 SC, Bayer CropScience, 200 g a.i./l) and deltamethrin (Decis® 2.5 EC Bayer CropScience, 25 g a.i./l) both commercialised for application in crops (vegetable and fruit). These two insecticides were selected because of their (1.) mode of action; (2.) data on agricultural application of insecticides in the area of Primorska region where *Nezara viridula* is present (Žežlina, I., 2007, personal communication); and (3.) data on annual sale of insecticides (for the years 2005 and 2006) acquired from the Phytosanitary Administration of the Republic of Slovenia (PARS) (Figure 7). The names of commercial products are simplified: Confidor 200 SC to imidacloprid and Decis 2.5 EC to deltamethrin.

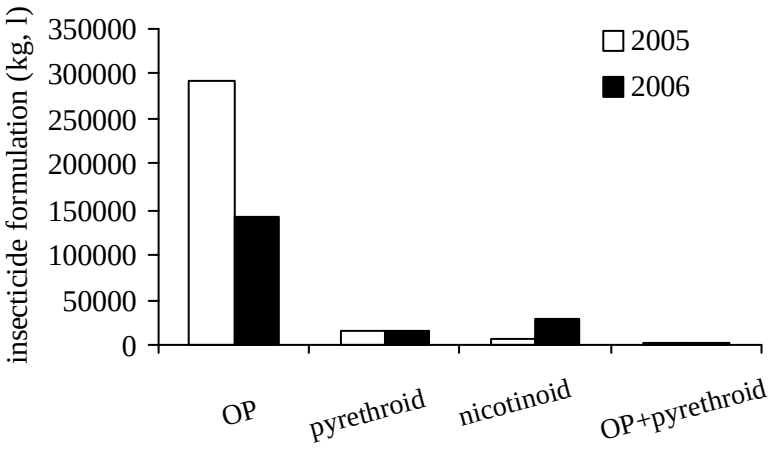

Figure 7. Annual sale of insecticides in Slovenia. Data for three groups of insecticide formulations (organophosphates (OP), pyrethroids and neonicotinoids) and formulations containing a mixture of OP and pyrethroids are shown for the years 2005 (white bars) and 2006 (black bars). Data were obtained from Phitosanitary Administration of the Republic of Slovenia.

3.5 Insecticide application

The effects of contact exposure to insecticides were assessed by topical application of the test solution on the ventral side of abdomen. Before insecticide application males were immobilized by soft tape extending over the thorax, to make sure the insecticide was not rubbed off from the body surface. Each male was afterwards treated with 1 µl of the specific insecticide solution using Hamilton Microliter™ Syringe. For each dose 15–20 males were used. Control males were immobilized and treated with 1 µl of distilled water. Mortality, physical condition and general responsiveness to FCS stimulation of males were observed 72 h (1, 2, 3, 24, 48, 72 h) after the control treatment.

3.6 Insecticide solutions

Imidacloprid and deltamethrin solutions were prepared in distilled water. The initial tested dose corresponded to the recommended field application doses (against insect pests of vegetables and fruits) were prepared in distilled water: 7.5 ml of Confidor in 10 l of water (corresponding to 150 ng of imidacloprid/µl) and 5 ml of Decis in 10 l of water (corresponding to 12.5 ng of deltamethrin/µl). Once prepared, the insecticide solutions were used for one month and were kept in the dark in refrigerator at 10°C. The half life of imidacloprid in distilled water is more than 30 days at 25°C in the dark (Bacey, 1999) and it is reduced to 1h when exposed to light (Abbott et al., 2008). During treatment we kept the insecticide solution in black bottles and within a box, in order to kept exposure to light at minimum. Solutions of the tested dose were stirred immediately prior to use.

3.7 Mortality and sublethal doses of imidacloprid and deltamethrin

Males were treated with initial tested dose (corresponding to field application dose). Insects were afterwards exposed to doses decreasing by factor 2 (or 4) until

observing mortality rate of 10% or less. If treatment at initial dose did not induce lethal effects, we increased the dose by factor 2 or until the LD_{50} was reached. Doses were expressed as ng of active ingredient per µl (ng/µl). The effects of each dose were tested on 20 males that were taken randomly from the rearing. Physical condition of insects and mortality counts were recorded 1, 2, 3, 24, 48 and 72 hours (h) after exposure to the insecticide. The sublethal doses, which induced poisoning symptoms in 10% (or less) of insects, were then used in further experiments and evaluate their impact on the specific behavioural. Insects were scored as affected when we observed at least one of the poisoning symptoms (locomotor difficulties, legs fully extended, paralysis, unable to right themselves when placed on their backs, tremor, uncoordinated movement, and/or self-cleaning behaviour).

3.8 Experiment 2-Effects of insecticides on male responsiveness to the female calling song (FCS)

We analysed the responses to the FCS stimulation after exposure to insecticide: playback experiments were conducted as described in experiment 1 (chapter 3.2, Figure 5, 6). Males were exposed to the stimulation program composed of the sequence of 35 artificially synthesised FCS and of 35 natural FCS pulse trains. To assess dose-dependant insecticide effects, we used four sublethal doses of imidacloprid (a neonicotinoid) determined in the previous experiment (150, 75, 38.5 and 18.5 ng/µl). For each tested dose we used different sexually mature males (chapter 3.1). Each male was exposed to the stimulation program before (pre-treatment trial) and after (post-treatment trial) exposure to the insecticide. Males which did not respond in the pre-treatment trial were eliminated from further experiments and replaced by a male of the same age. To assess the time-dependent effects of the imidacloprid males were tested at different time intervals of 1, 2, 3, 24, 48 and 72 h after exposure to the specific insecticide dose. For the dose of 150 ng/µl additional tests were performed at 96 and 120 h after the exposure. To avoid habituation, each male was tested only once a day, i.e. in one pair of pre- and post-treatment trial, therefore, different groups of males were used for trials of 1, 2, 3 h

(Figure 8), whereas the same group of males was used for trials of 24, 48 and 72 h. Time-dependent effects were evaluated for all four imidacloprid doses.

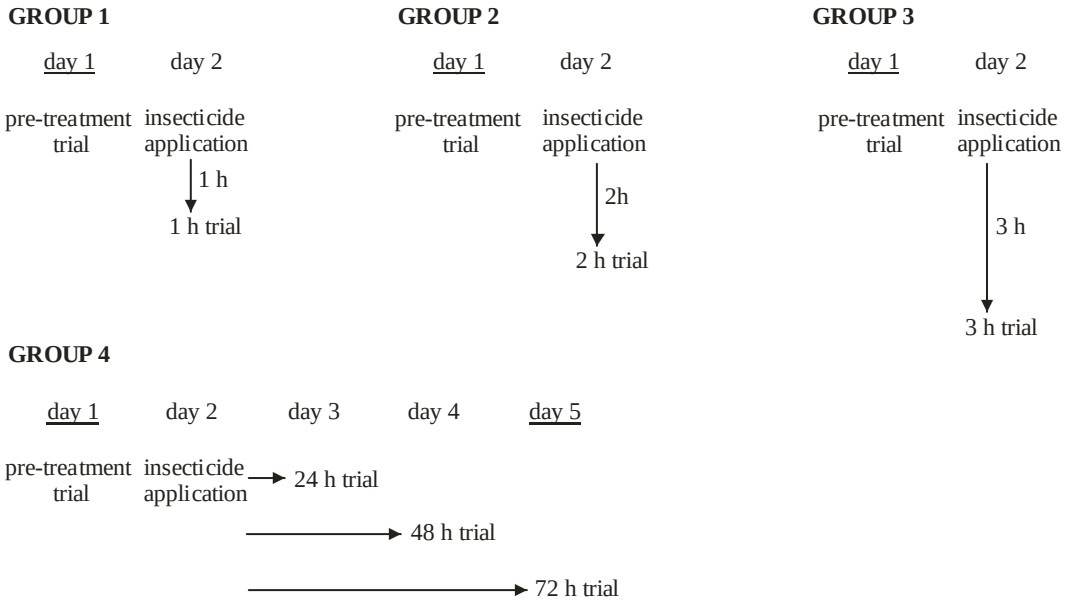

Figure 8: Scheme of time-dependent test trials within 72 h test period. Males were devided into four groups. A male was stimulated with female calling song before the exposure to insecticide (pre-treatment trial) and at the specific time interval after the exposure (post-treatment trial).

To compare the effects of two types of insecticides on the same behavioural pattern, we condacted another set of tests on males treated with sublethal dose of deltamethrin (pyrethroid insecticide, 1.5 ng/bug). Their responses, over 72 hours after the application of insecticide, were monitored and measured in the same way as for imidacloprid treated males described above.

Data analysis

For each chemical, cumulative mortality counts and percentage of affected bugs were determined at different time intervals after treatment (1, 2, 3, 24, 48, and 72 h) and compared with the control (1 µl of distilled water) (two-tailed Fisher's exact test, $P<0.05$).

The effects of insecticides on male responsiveness were evaluated by observing general responsiveness of males and analysing quality of the male response before

and after the exposure to the insecticide. First, we calculated the percentage of responsive males before and after the insecticide treatment. A male was scored as responsive when producing vibrational signals of male calling (MCS) and/or male courtship song (MCrS). We compared the percentage of responsive males in the pre-treatment trials with the percentage of responsive males at the corresponding post-treatment trial within 72 h test (two-sided Fisher's exact test). Furthermore, we evaluated the quality of MCrS response, produced during stimulation by 35 synthesised FCS pulse trains. Compared with MCS response, production of MCrS signals proved to be more species specific and important in the species recognition system (experiment 1). In order to evaluate the quality of the MCrS response we analysed five parameters (Figure 9): (1). duration of the MCrS pulse trains (expressed in ms); (2.) number of the pulse trains produced during FCS stimulation; (3.) the response latency 1 defined as number of FCS pulse trains needed to elicit the male response; (4.) the response latency 2 defined as time between onset of the FCS pulse train and onset of the subsequent MCrS response; and (5.) dominant frequency of the MCrS. For each male we calculated the mean value of the specific parameter of responses emitted in the pre-treatment trial and compared them with corresponding post-treatment trial (of specific time interval) (paired, two tailed T-test). We also analysed between-individual variation (coefficient of variation-CV, Gerhardt 1991).

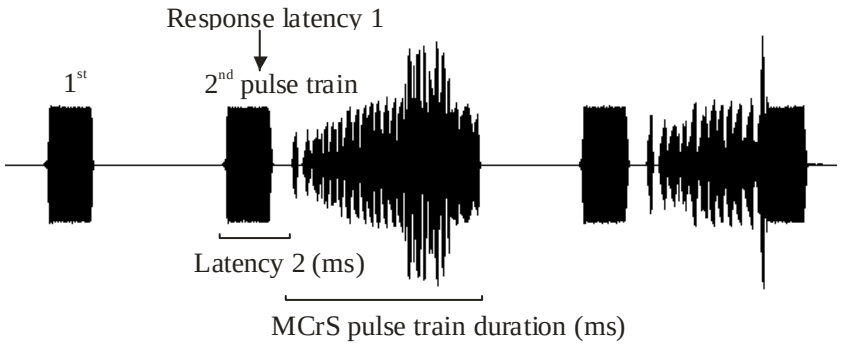

Figure 9: The quality of *Nezara viridula* male response defined by the following parameters: duration of the male courtship song (MCrS) pulse train, number of MCrS pulse trains (2 pulse trains shown), response latency 1 (number of female calling song (FCS) pulse trains needed to elicit male response), response latency 2 (time between the onset of FCS pulse train and subsequent MCrS response) and dominant frequency of MCrS pulse train.

3.9 Experiment 3-Effects of insecticides on male preference for the female calling song (FCS) pulse train duration and dominant frequency

We tested the effect of insecticide on male preference for pulse train duration (500, 600 ms) shorter from the mean value characteristic of the natural FCS and preference for dominant frequencies (90, 150 Hz) below and above frequency characteristic of the natural FCS. In experiment 1 males exhibited high preference (statistically not different from the preference for the natural song) for these values. We conducted four playback experiments (as in experiment 1; each male was stimulated with two sequences of 35 pulse trains, Figure 5a) and recorded the male responses before and after exposure to the insecticide. The first FCS sequence consisted of signals with modified pulse train duration or frequency (one parameter varied, while the others held constant) and the second sequence consisted of the natural FCS pulse trains. The latter was used to test daily responsiveness of the males and to test whether insecticide affects general responsiveness and ability to produce vibrational signals. We calculated the percentage of males responding to each type of stimulation (modified and/or natural FCS). High percentage of responding males indicated high preference for the specific stimulation. Male preference for specific stimulation in the pre-treatment trial was compared with the one from the post-treatment trial. Responses were considered significantly lower if the percentage of responding males in the post-treatment trials was significantly lower from the percentage of responding males in pre-treatment trial (Fisher's exact test, $P<0.05$).

We tested changes in male preferences for duration and frequency of the FCS pulse train after exposure to imidacloprid (neonicotinoid) and deltamethrin (pyrethroid). Males were treated with imidacloprid at dose of 38.5 ng/µl or with deltamethrin at dose of 1.5ng/µl. Playback experiments were conducted 1h after exposure to the insecticide. The treatment dose and the time interval were chosen on the basis of the results obtained in experiment 2 (Figure 24, 25, 28, 29). For each parameter value 20 males were taken randomly from the rearing and exposed to the specific stimulation program (pre-treatment trial). The next day males were treated (as in experiment 2)

with the specific insecticide and after 1 h they were exposed to the same stimulation program as in pre-treatment trial.

3.10 Experiment 4-Effects of insecticides on reproductive success of *N. viridula* males

In this experimental series we tested whether exposure to sublethal doses of imidacloprid and deltamethrin influence the subsequent reproductive success of treated males.

Sexually active adult males and females reared as described above (chapter 3.2) were used in this experiment. Individual males were exposed to the insecticide as in experiment 2. We carried out four treatments: (1). males were treated with imidacloprid at dose of 38.5 ng/µl; (2.) males were treated with deltamethrin: single treatment at dose of 1.5 ng/µl; (3.) males were treated with deltamethrin: multiple treatments at dose of 1.5 ng/µl (7 consecutive days); (4.) males were treated with 1 µl of distilled water (control treatment). Males were randomly paired with virgin females 24 h after exposure to the insecticide (in the third treatment 24h after the last application). Pairs were held in plastic cups, kept and fed at the same rearing conditions as in experiment 1, 2, 3 (chapter 3.2). In order to establish whether treated males were able to produce vibrational signals, we exposed them to the stimulation of FCS before we paired them with females. The reproductive success of treated males was evaluated following copulatory success, fecundity and fertility rate. Copulatory success was measured by percentage of copulating males, the number of copulation/male and time spend in copula. Fecundity and fertility rate was measured by percentage of ovipositing females, the number of egg masses/female, the number of eggs/egg mass and the number of adults of the first filial generation (F1). All parameters were compared between control and insecticide treated males. The proportion of copulating males and ovipositing females were compared using two-tailed Fisher's exact test. Other parameters were compared using Kruskal Wallis and post-hoc Steel test. In all cases statement of statistical significance implies $P<0.05$.

4 RESULTS

Within the present study we conducted four experiments. Results are shown separately for each experiment.

4.1 Experiment 1-Male preference for the temporal and spectral parameters of the female calling song (FCS)

In the experiment 1 we investigated male responses to artificially synthesised FCS stimuli of different temporal and spectral parameter values. One parameter was varied at the time and the others were held constant at the mean value characteristic of the natural FCS (Table 4–7, Figure 10–16).

4.1.1 Male preference for the FCS pulse train duration

In the first experimental series we measured male responsiveness to stimulation with artificially synthesised FCS pulse trains of variable durations (400–9000 ms, Figure 5c, 6a) and of interval and dominant frequency values characteristic of the natural FCS (i.e. 1900 ms, 105 Hz, respectively). The maximum responsiveness was recorded at the pulse train duration of 700 ms (92.8%, 13/14), characteristic of the natural FCS (reference value, Figure 10). When changing duration, below or above the reference value, the percentage of responding males decreased. While responsiveness was greatly reduced towards shorter stimuli, responsiveness showed a weak roll-off towards longer stimuli. A decrease of the reference duration by 43% (i.e. 400 ms) significantly decreased responsiveness by 37% (Fisher's exact test, $P<0.05$); with increasing of the reference value by 43% (i.e. 1000 ms) the responsiveness decreased only by 15% (Fisher's exact test, $P=0.355$ NS; Figure 10a). The effective range of the stimulus pulse train duration was observed between 600 ms and 1000 ms (duration values which elicited a response not significantly different from the response to the reference, Fisher's exact test, $P>0.05$). Males exhibited less

pronounced second response peak to the stimulus of the natural FCrS (FCrS vs. 700 ms: Fisher's exact test, P=0.064 NS) and to synthesised FCS stimulus of 4000 ms duration (4000 vs. 700 ms: two-tailed Fisher's exact test, P<0.05). Third increase in the responsiveness occurred at the stimulus of 7000 ms duration, however, it did not reach the level observed at the reference duration (Fisher's exact test, P<0.05). None of the tested males responded to very short (200 ms) pulse train duration. There was no correlation between the emission of MCS and variable duration values: between 5 and 11% of males responded to all stimuli only with MCS. However, when exposed to stimulus of the natural FCrS, more than half of the responding males (62.5%, 10/16) emitted only MCS (Figure 10a).

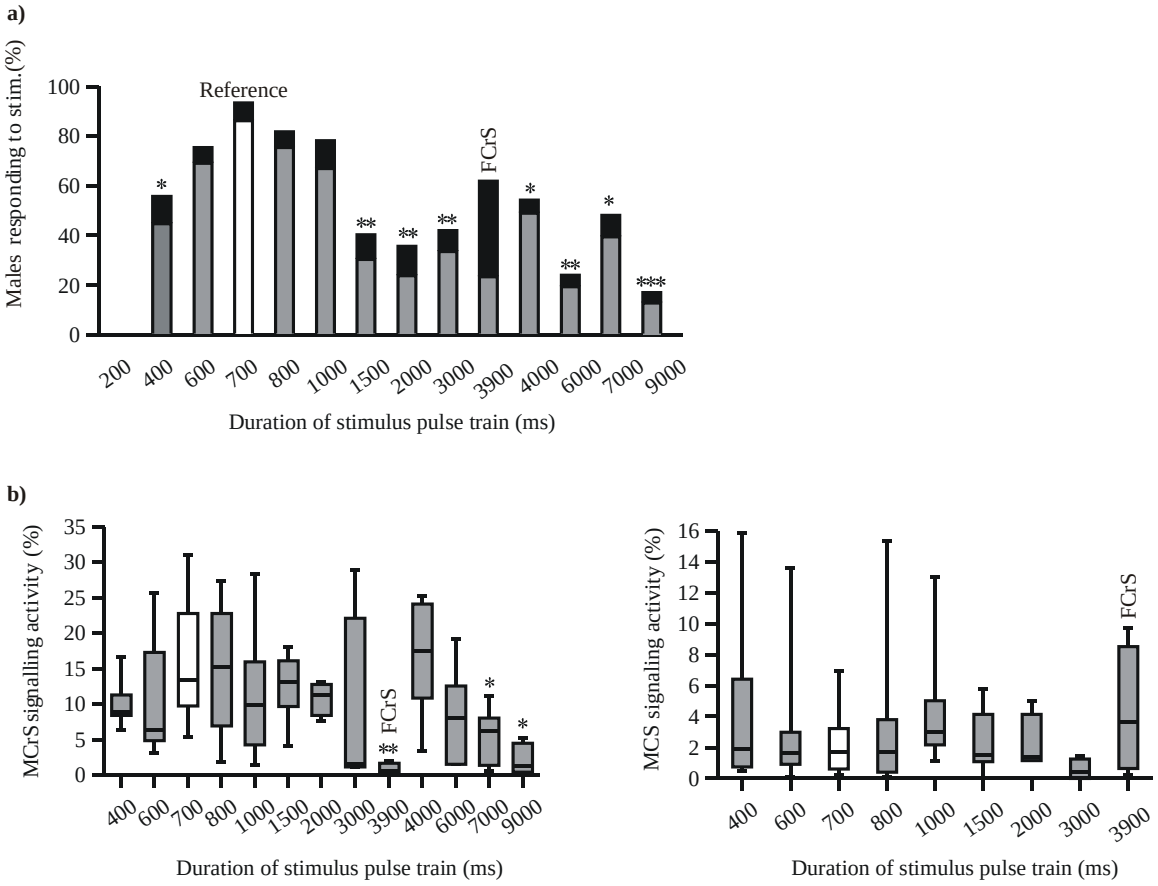

Figure 10: Vibrational response of *Nezara viridula* males exposed to the synthesised female calling song (FCS) of variable pulse train duration (400–9000 ms) and the natural female courtship song (FCrS; 3900 ms). (a) Percentage of males responding to stimuli of variable duration (grey bars: percentage of males responding with male courtship song (MCrS) and male calling song (MCS); black bars: percentage of males responding with MCS only). Asterisks indicate the responsiveness that is significantly lower from the responsiveness to the reference value (white bar; 700 ms, i.e. characteristic of the natural FCS; Fisher's exact test * P<0.05; **, P<0.01, ***, P<0.001). (b) MCrS (left) and MCS (right) signalling activity of males exposed to stimuli of variable duration. Reference value is indicated with the white bar. Asterisks indicate significant difference between signalling activity of males exposed to the reference value stimulus (700 ms) and to the stimuli below and above the reference value (Steel test * P<0.05, ** P<0.01, *** P<0.001). The exact median values and significance levels are given in Table 4.

Male responses to different stimuli were analysed afterwards and their MCrS and MCS signalling activity was evaluated (Table 4, Figure 10b). High MCrS signalling activity (measured as the sum of durations of all emitted MCrS) was observed at the stimuli of 700 ms (Me=13.43%, reference value) and 800 ms (15.21%, Table 4, Figure 10b left, 800 ms vs. 700 ms: Steel test: P>0.05). The signalling activity decreased either by shortening or prolonging of the reference value. Significantly lower signalling activity was observed at stimulus of the natural FCrS (Me=0.61%, Steel test, P<0.01). Signalling activity at synthesised stimulus of 3000 ms duration was low as well (Me=1.45%), but only three males responded to this stimulus,

compared to reference value, no significant difference was measured (Table 4). Furthermore, the variability of signalling activity at 3000 ms was very high, ranging from 28.88 to 0.92% (Figure 10b left). At stimuli of 4000 and 6000 ms durations MCrS signalling activity (Me=17.48, 18.01, respectively) again reached the level observed at the reference stimulus (4000 vs. 700 ms: Steel test, P=1.000 NS; 6000 vs. 700 ms: Steel test, P=0.266 NS). When males were stimulated with even longer pulse trains (7000, 9000 ms), signalling activity significantly decreased again (Me=6.23, 1.26%, respectively, 7000 vs. 700 ms: Steel test, P<0.05; 9000 vs. 700 ms: Steel test, P<0.05). At stimuli of very short and very long FCS pulse trains reduced variability of MCrS signalling activity was observed (Figure 10b).

Table 4: Male courtship song (MCrS) and the male calling song (MCS) signalling activity at synthesised stimuli of FCS pulse trains of variable durations (400–9000 ms) and natural female courtship song (FCrS). Signalling activity was measured as the sum of durations of all emitted MCrS or MCS. Given are median values and comparison of signalling activity at the reference pulse train duration (i.e.700 ms, characteristic of the natural FCS) and signalling activity at durations below and above the reference. Significant difference (Steel test, P<0.05) is indicated with bold letters. N represents the number of males responding to specific stimulus value. For further details see Figure 10.

Duration of the FCS pulse train (ms)	MCrS signalling activity (%)		MCS signalling activity (%)	
	Median	P value	Median	P value
700	13.43 N=12		1.69 N=12	
400	8.82 N=8	0.565 NS	1.89 N=10	0.990 NS
600	6.42 N=11	0.822 NS	1.81 N=12	1.000 NS
800	15.21 N=12	1.000 NS	2.15 N=13	1.000 NS
1000	9.80 N=12	0.719 NS	3.17 N=11	0.414 NS
1500	13.13 N=6	1.000 NS	1.50 N=8	1.000 NS
2000	11.25 N=4	0.998 NS	1.39 N=4	1.000 NS
3000	1.45 N=3	0.660 NS	0.65 N=3	0.863 NS
3900 (natural FCrS)	0.61 N=7	**0.037**	3.66 N=12	0.992 NS
4000	17.48 N=6	1.000 NS	0.31 N=5	0.076 NS
6000	8.01 N=10	0.431 NS	0.21 N=4	0.219 NS
7000	6.23 N=4	**0.031**	0.43 N=5	0.430 NS
9000	1.26 N=4	**0.037**	0.68 N=2	0.680 NS

When varying duration of FCS pulse trains no significant differences were observed in the median values of the MCS signalling activity (measured as the sum of durations of all emitted MCS). Maximum MCS signalling activity was measured at FCrS stimulus (3.66%) (Table 4, Figure 10b right). Variability of signalling activity was high for all stimuli (except for stimuli 1500–3000 ms). The MCS signalling activity was evaluated only for stimuli of which duration ranged between 400 and

3000 ms and for the FCrS stimulus. At longer durations, complete overlapping of stimuli and MCS signals occurred, therefore precise analyses of MCS parameters was not possible.

4.1.2 Male preference for the FCS inter-pulse train interval (interval)

In this experimental series we analysed which inter-pulse train interval (interval) values are preferred by males. We tested male responsiveness to stimulation with artificially synthesised FCS of varying interval values (300–10000 ms, Figure 5c, 6b); duration and dominant frequency of the pulse trains were kept constant at values characteristic of the natural FCS (Figure 11). The maximum responsiveness of males was recorded at 1900 ms interval (92%, 23/25, Figure 11a), characteristics of the natural FCS (reference value). Either increasing or decreasing the interval reference value decreased male responsiveness. The effective range of the stimulus interval was observed between 1500 and 3000 ms (Figure 11a). The decrease in response toward shorter interval values (<1900 ms) was constant, with no secondary peak. At longer values (>1900 ms) secondary peak occurred at 7000 ms stimulus (95%, 19/20) where the level of responsiveness slightly exceeded the level of responsiveness to reference value (Fisher's exact test, P=1.000 NS). Steeper roll-off towards shorter interval values was observed. With 50% change of the interval, below (i.e. 1000 ms) or above (i.e. 4000 ms) the reference, the responsiveness significantly decreased by 62% for the shorter (1000 ms vs. 1900 ms: Fisher's exact test: P<0.001) and only by 20% for the longer value (4000 ms vs. 1900 ms: Fisher's exact test: P<0.05).

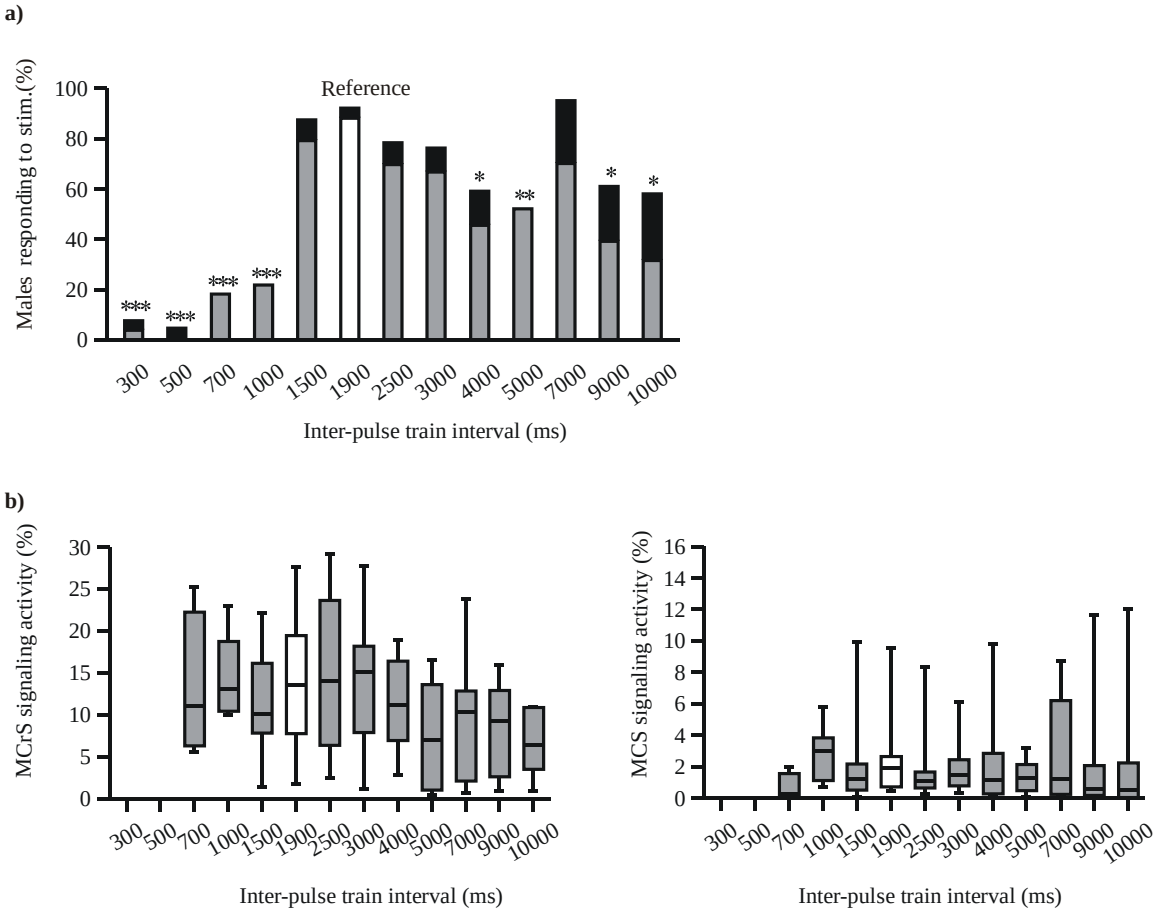

Figure 11: Vibrational response of *Nezara viridula* males exposed to the synthesised female calling song (FCS) of variable inter-pulse train interval (interval) (300–10000 ms). (a) Percentage of males responding to stimuli of variable interval (grey bars: percentage of males responding with male courtship song (MCrS) and male calling song (MCS); black bars: percentage of males responding with MCS only). Asterisks indicate the responsiveness that is significantly lower from the responsiveness to the reference value (white bar; 1900 ms, i.e. characteristic of the natural FCS; Fisher's exact test * P<0.05; **, P<0.01, ***, P<0.001). (b) MCrS (left) and MCS (right) signalling activity of males exposed to stimuli of variable interval. Reference value is indicated with the white bar. Asterisks indicate significant difference between signalling activity of males exposed to the reference value stimulus (1900 ms) and to the stimuli below and above the reference value (Steel test * P<0.05, ** P<0.01, *** P<0.001). The exact median values and significance levels are given in Table 5.

Additionally, we evaluated signalling activity of responding males (Table 5, Figure 11b). The median value of MCrS signalling activity decreased with intervals shorter than reference value (1900 ms), but the differences were not statistically significant (Steel test, NS, Table 5, Figure 11b right). Only one male responded with MCrS to 300 ms stimulus and its signalling activity was higher (22.16%) than the one at the reference stimulus. Statistics and box plot were not performed for this male. None of the tested males responded with MCrS response to the stimulus of 500 ms interval. With prolongation of the reference value no significant difference in median values

of signalling activity were observed (Steel test, NS Table 5, Figure 11b). But prolongation of the reference value reduced variability of signalling activity.

The median values of MCS signalling activity ranged between 0.25 and 3.17% according to different stimulus, with maximum MCS value at 1000 ms interval (Table 10, Figure 11b left). No significant differences were observed between MCS signalling activity of males exposed to reference interval value and values shorter or/and longer from the reference.

Table 5: Male courtship song (MCrS) and the male calling song (MCS) signalling activity at synthesised stimuli of FCS pulse trains of variable inter-pulse train interval (interval) (300–10000 ms). Signalling activity was measured as the sum of durations of all emitted MCrS or MCS. Given are median values and comparison of signalling activity at the reference interval (i.e.1900 ms, characteristic of the natural FCS) and signalling activity at intervals below and above the reference. Significant difference (Steel test, P<0.05) is indicated with bold letters. N represents the number of males responding to specific stimulus value. For further details see Figure 11.

Interval (ms)	MCrS signalling activity (%)		MCS signalling activity (%)	
	Median	P value	Median	P value
1900	13.61 N=20		2.11 N=19	
300	22.16 N=1	/	0.91 N=2	0.641 NS
500	/	/	0.54 N=1	
700	10.97 N=4	1.000 NS	0.25 N=4	0.171 NS
1000	14.34 N=5	0.999 NS	3.17 N=5	0.969 NS
1500	10.24 N=19	0.998 NS	1.19 N=21	0.844 NS
2500	13.98 N=16	0.999 NS	1.17 N=17	0.840 NS
3000	15.19 N=13	1.000 NS	1.67 N=15	0.999 NS
4000	11.16 N=10	0.993 NS	1.58 N=3	0.995 NS
5000	6.96 N=12	0.141 NS	1.27 N=10	0.998 NS
7000	10.26 N=14	0.327 NS	1.19 N=16	0.753 NS
9000	9.31 N=9	0.681 NS	0.66 N=13	0.538 NS
10000	6.37 N=6	0.206 NS	0.51 N=8	0.500 NS

4.1.3 Male preference for duty cycle of the FCS

In this experimental series we tested male responses to stimulation of artificially synthesised FCS of constant duty cycle (27%, characteristic of the natural FCS) obtained by simultaneously decreasing or increasing the duration and interval of FCS pulse trains (Figure 5c, 6c). The maximum responsiveness (95.8%, 23/24) was

observed at the duty cycle of 27% obtained by 800 ms stimulus duration and 2109 ms interval. Males exhibited 81.5% (22/27) responsiveness when exposed to stimuli with 27% duty cycle of pulse train duration (700 ms) and interval (1872 ms) values, which are characteristic of the natural FCS (i.e. reference value; Figure 12a; 800 and 2109 ms vs. 700 and 1872 ms: Fisher's exact test: P=0.197 NS). Duty cycle of 27% still elicited no significant difference in responsiveness, when defined by duration and interval values of 600 and 1582 ms, 1000 and 2636 ms, respectively (600 and 1582 vs. 700 and 1872, Fisher's exact test: P=1.000 NS; 1000 and 2636 vs. 700 and 1872, Fisher's exact test: P=0.526 NS). Duty cycle of 27% became significantly less attractive to males when it was obtained by approximately half (400 and 1054 vs. 700 and 1872: Fisher's exact test: P<0.001) or twice the reference duration and interval (1500 and 3955 vs. 700 and 1872: Fisher's exact test: P<0.01). None of the tested males responded to 27% duty cycle obtained by very short values of both, duration and interval (i.e. 200 ms and 527 ms, respectively). However, when stimulated with 700 ms stimulus duration (characteristic of the natural FCS) and very short intervals (300 and/or 500 ms, while interval characteristic of the natural FCS is 1900 ms), males responded to stimuli, but the responsiveness was very low 7.7% (2/26) and 4.7% (1/21), respectively (see chapter 4.1.2, Figure 12a).

Results on measuring preference for pulse train duration and interval (Figure 10a, 11a) indicated the effective range of duty cycle as well. Prolonging of the pulse train duration (with constant interval), resulted in increasing duty cycle. No significant difference in responsiveness was observed when males were exposed to reference stimulus and to stimuli of duty cycle ranging from 24% and 34.5%, obtained by equal interval (1900 ms) and variable duration (600–1000 ms) (Fisher's exact test: P>0.05, NS). On the other hand, prolonging of the interval (with constant pulse train duration) resulted in decreasing duty cycle. No significant difference in responsiveness was observed when males were exposed to reference stimulus and to stimuli of duty cycle ranging from 32% and 18.9%, defined by equal duration (700 ms) and variable interval (1500–3000 ms) (Fisher's exact test: P>0.05, NS).

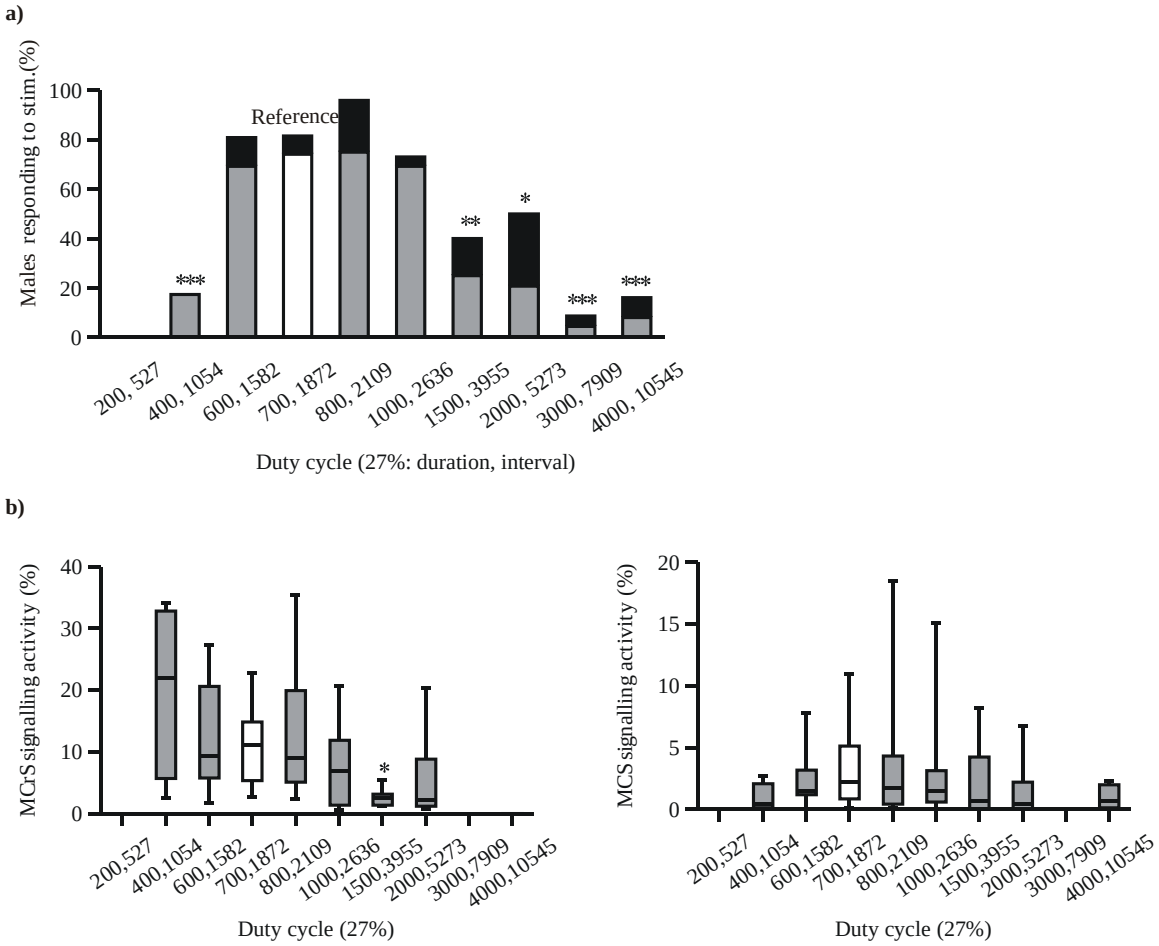

Figure 12: Vibrational response of *Nezara viridula* males exposed to the synthesised female calling song (FCS) of 27% duty cycle defined by variable duration (200–4000 ms) and inter-pulse train interval (interval) (527–10545 ms). (a) Percentage of males responding to variable stimuli characteristics (grey bars: percentage of males responding with male courtship song (MCrS) and male calling song (MCS); black bars: percentage of males responding with MCS only). Asterisks indicate the responsiveness that is significantly lower from the responsiveness to the reference value (i.e. 27% duty cycle defined by 700 ms duration and 1900 ms interval, characteristic of the natural FCS (white bar), Fisher's exact test * P<0.05; **, P<0.01, ***, P<0.001). (b) MCrS (left) and MCS (right) signalling activity of males exposed to stimuli of 27% duty cycle defined by variable duration and interval values. Reference value is indicated with the white bar. Asterisks indicate significant difference between signalling activity of males exposed to the reference value and to the stimuli of values below and above the reference (Steel test * P<0.05, ** P<0.01, *** P<0.001). The exact median values and significance levels are given in Table 6.

MCrS and MCS signalling activity of responding males is shown in the Table 6 and Figure 12b. The maximum MCrS signalling activity (Me=22.02) was exhibited at 27% duty cycle obtained by 400 ms duration and 1054 ms interval (Figure 12b right). The number of responding males to this stimulus was low (N=4), the variation in signalling activity was high (Me ranging from 2.5% to 34.1%) and compared to signalling activity of males exposed to reference stimulus, the difference was not statistically significant (Steel test: P>0.05, NS). Stimuli of 27% duty cycle defined

by duration and interval above their reference values induced a decline in the MCrS signalling activity. However, the difference was statistically significant only at 1500 ms stimulus duration and 3955 ms interval (Steel test, P <0.05, Table 6, Figure 12), with relatively small variation in signalling activity. Very low signalling activity was exhibited at stimuli of even longer duration and interval (Me=0.35% at 3000 and 7909 ms; Me=1.58% at 4000 and 10545 ms, respectively). The number of males that responded to these stimuli was very low (N=1, 2 respectively), and the statistical analysis does not show any significant difference when compared with the reference stimulus. The box plot is not shown because of very few responding males.

Table 6: Male courtship song (MCrS) and the male calling song (MCS) signalling activity at synthesised stimuli of FCS pulse trains of 27% duty cycle defined by variable pulse train duration and inter-pulse train interval (interval). Signalling activity was measured as the sum of durations of all emitted MCrS or MCS. Given are median values and comparison of signalling activity at the reference value (i.e. 700 ms duration, 1872 ms interval), characteristic of the natural FCS) and signalling activity at stimuli of duration and interval values below and above the reference. Significant difference (Steel test, P<0.05) is indicated with bold letters. N represents the number of males responding to specific stimulus value. For further details see Figure 12.

Duration, interval of FCS pulse trains (ms)	MCrS signalling activity (%)		MCS signalling activity (%)	
	Median	P value	Median	P value
700, 1872	11.39 N=17		2.19 N=20	
200, 527	/	/	/	/
400, 1054	22.02 N=4	0.803 NS	0.40 N=4	0.641 NS
600, 1582	9.32 N=18	1.000 NS	1.50 N=20	0.171 NS
800, 1209	9.20 N=19	1.000 NS	1.68 N=23	0.969 NS
1000, 2636	6.96 N=18	0.439 NS	1.50 N=16	0.844 NS
1500, 3955	2.89 N=5	**0.039**	0.68 N=8	0.840 NS
2000, 5273	2.09 N=6	0.141 NS	0.43 N=10	0.999 NS
3000, 7909	0.35 N=1			0.995 NS
4000, 10545	1.58 N=2	0.159 NS	0.68 N=4	0.998 NS

The median values of MCS signalling activity ranged between 0.40 and 2.19% at different stimuli, with maximum MCS value at 27% duty cycle obtained by duration and interval values characteristic of the natural FCS. No significant differences were observed between MCS signalling activity of males exposed to reference duration and interval values and values below or/and above the reference values (Steel test: P>0.05, NS, Table 6, Figure 12b left). The variability of MCS signalling activity was very high, but decreased with duration and interval values below or above the reference values.

4.1.4 Male preference for repetition time of the FCS pulse trains

In this experimental series, we tested male responsiveness to stimuli of different repetition time (Figure 5c, 6e). The maximum responsiveness (80–100%) was measured at the repetition time characteristic of the natural FCS (reference value, 2600 ms) (Figure 13). However, the repetition time of 2600 ms became significantly less attractive (only 20% of males responded, Fisher's exact test: $P<0.01$) obtained by duration and interval values outside their effective range (for duration 600–1000 ms, Figure 10a; for interval 1500–3000 ms, Figure 11a). Males preferred longer over shorter repetition time values. Decreasing the repetition time induced steep and constant decrease in responsiveness (below 40% of males responded to repetition time shorter than 2600 ms), and by increasing the repetition time, the level of responsiveness decreased gradually and depended more on the attractiveness of specific duration and interval values. Interval was the least limiting parameter: stimuli of long interval value (~9000ms) and 700 ms duration (characteristic of the natural FCS) elicited response in 60% of males, whereas, when both of the parameters were long, the responsiveness decreased below 60%.

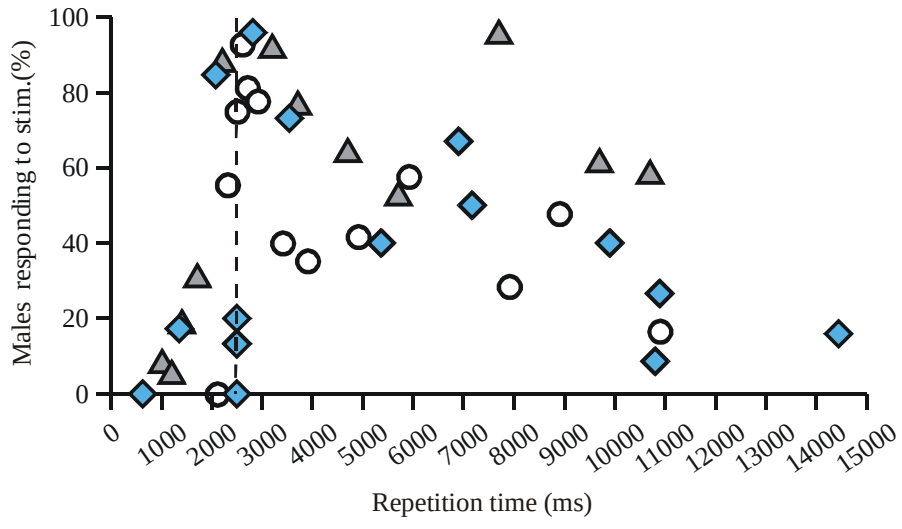

Figure 13: Percentage of *Nezara viridula* males responding to female calling song (FCS) stimuli of variable repetition time values. Different values were obtained by varying duration with interval held constant (1900ms) (circles); by varying interval with duration held constant (700ms) (triangles); by simultaneously varying duration and interval (diamonds). The value of repetition time characteristic of the natural FCS is indicated by dashed line.

Results on measuring preference for pulse train duration and interval (Figure 10a, 11a) indicated the effective range of repetition time as well. Prolonging of the pulse train duration (with constant interval) resulted in increasing repetition time. No significant difference in responsiveness was observed, when males were exposed to reference stimulus and to stimuli of repetition time ranging from 2500 and 2900 ms and obtained by equal interval (1900 ms) and duration values between 600 and 1000 ms (Fisher's exact test: $P>0.05$, NS). Prolonging of the interval between pulse trains (with constant duration) resulted in increasing repetition time as well. No significant difference in responsiveness was observed when males were exposed to reference stimulus and to stimuli of repetition time ranging from 2200 and 3700 ms, defined by equal duration (700 ms) and interval values between 1500 and 3000 ms (Fisher's exact test: $P>0.05$, NS).

4.1.5 Male preference for dominant frequency of the FCS pulse trains

We tested male responsiveness to FCS stimuli of variable pulse train dominant frequency (Figure 6d). Males exhibited unimodal response function to different dominant frequencies and constant temporal characteristics (Figure 14). The maximum responsiveness (91.3%, 21/23) was observed at stimulus of the dominant frequency, characteristic of the natural FCS (i.e. 105 Hz, reference value). The effective range of dominant frequency lies between 90 and 180 Hz (Figure 14a). None of the tested males responded to stimuli of dominant frequency below 70 and above 250 Hz. Decreasing and increasing the reference frequency resulted in responsiveness decrease. The roll-off towards frequencies below the reference value was steeper than to the ones above the reference: with 15% decrease of the reference (i.e. 90 Hz), responsiveness decreased to 70% (14/20, Fisher's exact test: $P=0.263$, NS), and with 15% increase (i.e. 120 Hz) the responsiveness decreased to 84% (21/25, Fisher's exact test: $P=1.000$, NS). When the reference value changed for 40%, the stimuli became significantly less attractive. The differences in responsiveness were statistically significant for both, 40% decrease in frequency (i.e. 70 Hz, Fisher's exact test, $P<0.001$) and 40% increase (i.e. 200 Hz, Fisher's exact test, $P<0.05$).

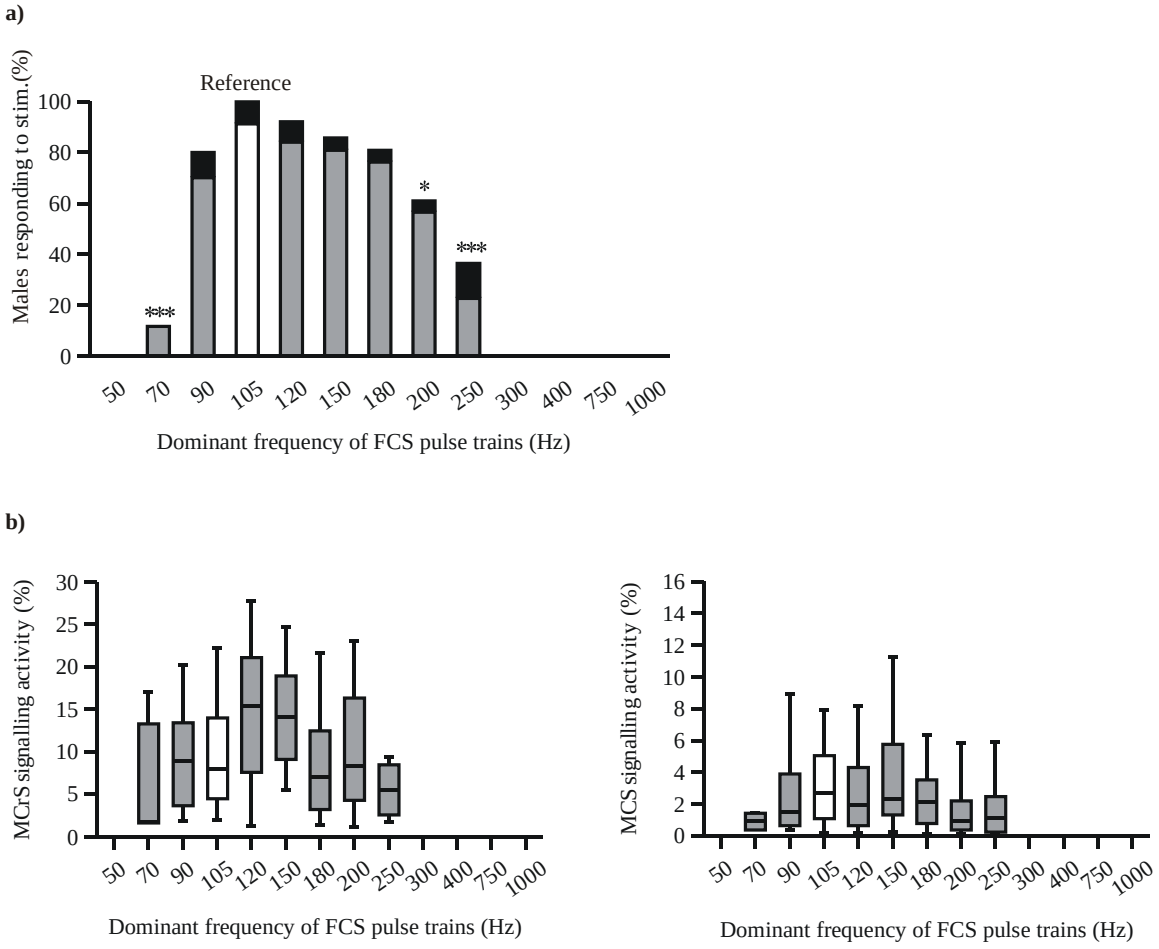

Figure 14: Vibrational response of *Nezara viridula* males exposed to the synthesised female calling song (FCS) of variable dominant frequency (50–1000 Hz). (a) Percentage of males responding to stimuli of variable frequency (grey bars: percentage of males responding with male courtship song (MCrS) and male calling song (MCS); black bars: percentage of males responding with MCS only). Asterisks indicate the responsiveness that is significantly lower from the responsiveness to the reference value (white bar; 105 Hz, i.e. characteristic of the natural FCS; Fisher's exact test * P<0.05; **, P<0.01, ***, P<0.001). (b) MCrS (left) and MCS (right) signalling activity of males exposed to stimuli of variable dominant frequency. Reference value is indicated with the white bar. Asterisks indicate significant difference between signalling activity of males exposed to the reference value stimulus (105 Hz) and to the stimuli below and above the reference value (Steel test * P<0.05, ** P<0.01, *** P<0.001). The exact median values and significance levels are given in Table 7.

Unimodal response function was observed when analysing the MCrS and MCS signalling activity (Figure 14b). Interestingly, the highest median value of MCrS activity was observed at the stimulus of 120 Hz (Me=16.4) and not at the reference value (105 Hz) which elicited the maximum male responsiveness. However, the difference was not statistically significant (Steel test, P>0.05, NS, Table 7, Figure 14b right). Stimulus of 150 Hz also exhibited higher MCrS signalling activity from that exhibited at the reference, but the difference was not statistically significant (Steel test, P>0.05, NS, Table 7). MCrS signalling activity was very low at the

stimulus of 70 Hz (Me=1.7%). The number of responding males was low (N=4) and statistical analysis does not show the significant difference when compared with signalling activity of males exposed to the reference frequency (Table 7). The highest MCS signalling activity (Figure 14b left) was observed at stimulus of reference frequency value. Signalling activity of MCS response did not significantly differ between stimuli of different frequency values (Table 7).

Table 7: Male courtship song (MCrS) and the male calling song (MCS) signalling activity at synthesised stimuli of FCS pulse trains of variable dominant frequency. Signalling activity was measured as the sum of durations of all emitted MCrS or MCS. Given are median values and comparison of signalling activity at the reference frequency (i.e. 105 Hz, characteristic of the natural FCS) and signalling activity at frequencies below and above the reference. Significant difference (Steel test, P<0.05) is indicated with bold letters. N represents the number of males responding to specific stimulus value. For further details see Figure 14.

Dominant frequency of FCS pulse trains (Hz)	MCrS signalling activity (%)		MCS signalling activity (%)	
	Median	P value	Median	P value
105	8.26 N=19		2.73 N=19	
50	/	/	/	/
70	1.70 N=4	0.789 NS	0.90 N=4	0.264 NS
90	8.88 N=12	0.999 NS	1.50 N=12	0.818 NS
120	16.60 N=19	0.179 NS	1.98 N=21	0.999 NS
150	14.18 N=15	0.145 NS	2.29 N=16	0.999 NS
180	7.36 N=15		2.14 N=16	0.879 NS
200	8.28 N=12	0.981 NS	0.90 N=13	0.334 NS
250	5.53 N=4	0.956 NS	1.45 N=5	0.877 NS
300	/	/	/	/
400	/	/	/	/
750	/	/	/	/
1000	/	/	/	/

4.1.6 Pre-pulse of the FCS pulse trains

Two types of pulse trains of *N. viridula* FCS were described (Čokl et al. 2000). The broad-band pulse trains are composed of separate pulses and narrow-band pulse trains of a short pre-pulse (133 Hz frequency, 80 ms duration, 0.02 mm/s velocity) followed by a longer pulse. This experiment was conducted to determine the relevance of the FCS pre-pulse (Figure 5b) for the calling song recognition. The pre-pulse velocity was changed between 0.02 and 0.416 mm/s (Figure 15) and its frequency between 70 and 200 Hz (Figure 16). High percentage (87.5%, 21/24) of

males responded to stimulus of the synthesised FCS with pre-pulse velocity characteristic of the natural FCS (0.02 mm/s, reference value, Figure 15a). The percentage of responding males significantly decreased (56%, 14/25) when males were stimulated with the FCS signals without the pre-pulse (Fisher's exact test, $P<0.05$). On the other hand, compared to reference value, no significant differences were recorded with increasing of the pre-pulse velocity (0.14 and 0.41 mms/) (Fisher's exact test: $P=1.000$, 0.609, respectively, Figure 15a).

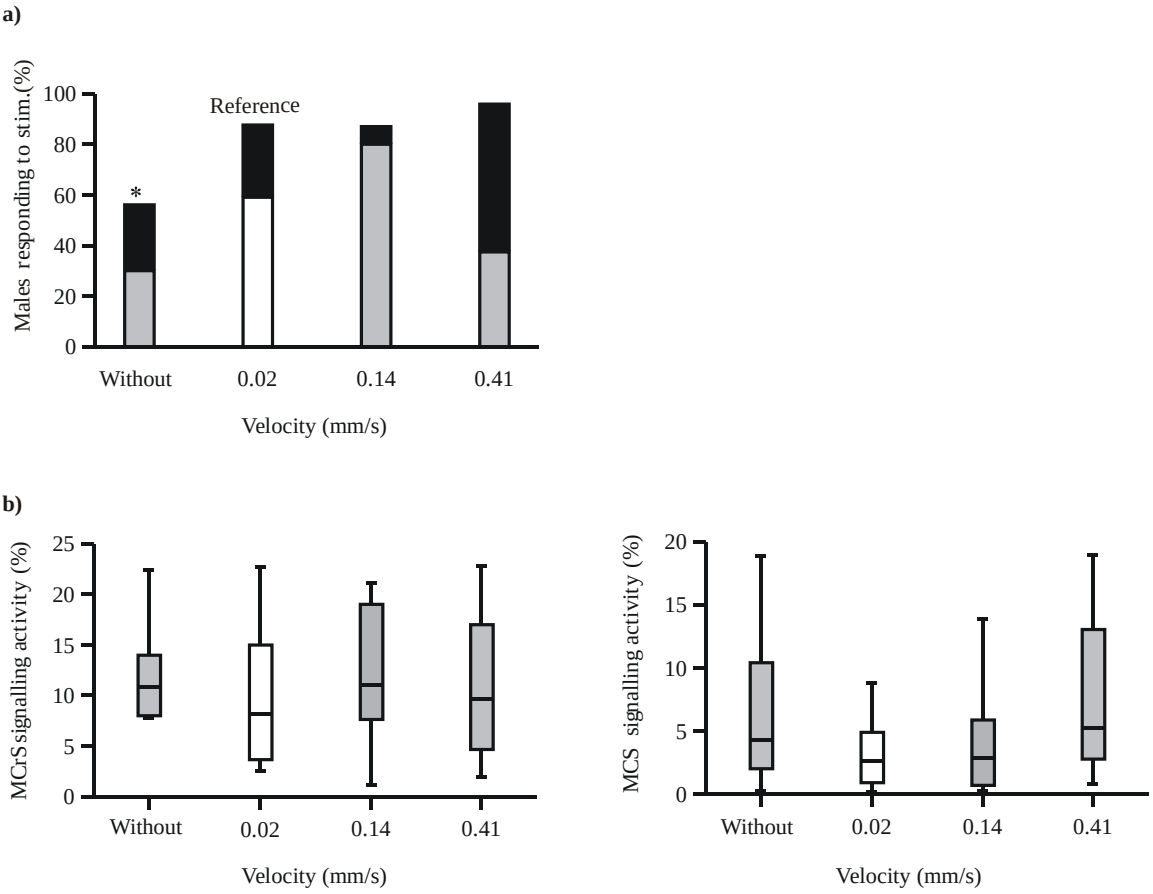

Figure 15: Vibrational response of *Nezara viridula* males exposed to the synthesised female calling song (FCS) of variable pre-pulse velocity (0.02–0.416 mm/s). (a) Percentage of males responding to stimuli of variable velocity (grey bars: percentage of males responding with male courtship song (MCrS) and male calling song (MCS); black bars: percentage of males responding with MCS only). Asterisks indicate the responsiveness that is significantly lower from the responsiveness to the reference value (white bar; 0.02 mm/s, i.e. characteristic of the natural FCS; Fisher's exact test * P<0.05). (b) MCrS (left) and MCS (right) signalling activity of males exposed to stimuli of variable pre-pulse velocity. Reference value is indicated with the white bar.

High percentage (72%-93%) of responding males was observed at all tested stimuli of variable pre-pulse frequency values and no differences were observed between

reference value (133 Hz) and values below or above the reference (Figure 16a, Fisher's exact test, P>0.05, for all tested values). Relatively high percentage of males responded only with MCS to stimuli of pre-pulse frequencies below the reference value (70-105 Hz). No correlation was observed between MCrS or MCS signalling activity with either velocity or frequency variation of the pre-pulse (Figure 15b, 16b; Steel test, P>0.05 for all tested values).

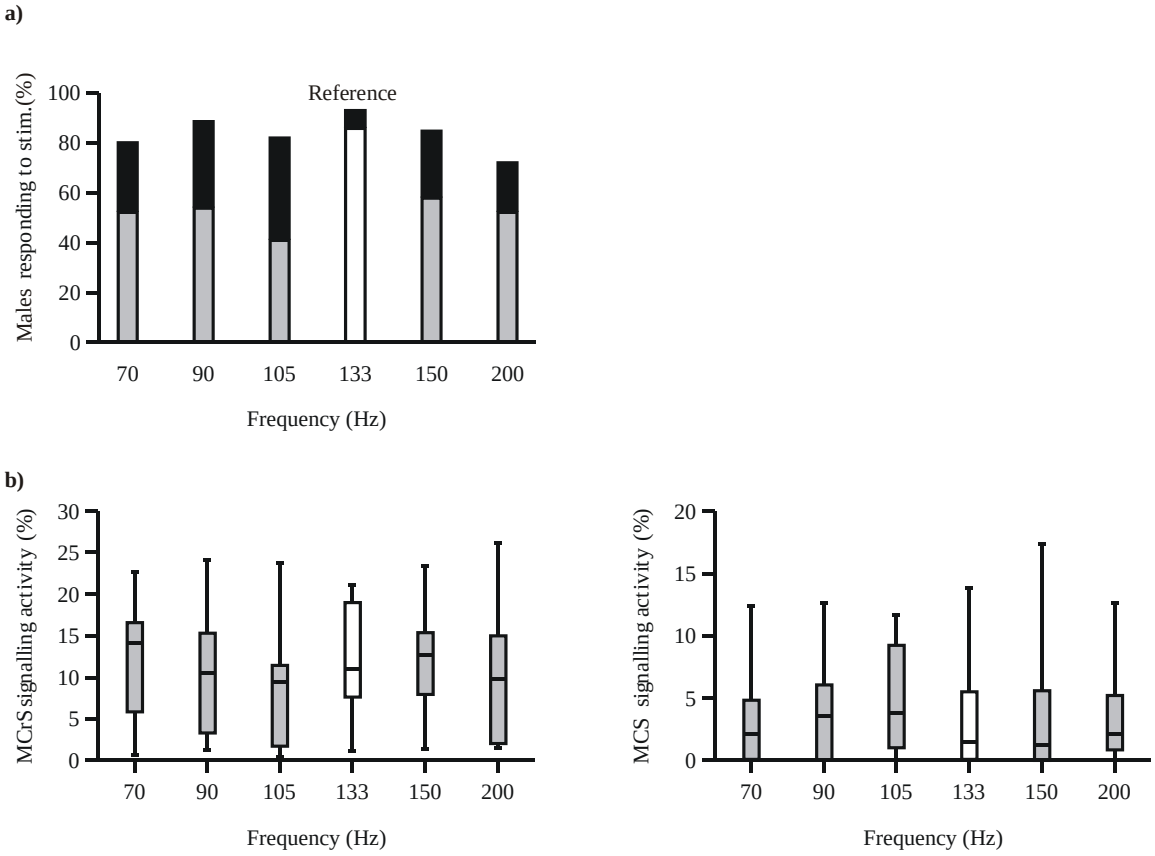

Figure 16: Vibrational response of *Nezara viridula* males exposed to the synthesised female calling song (FCS) of variable pre-pulse frequency. (a) Percentage of males responding to stimuli of variable frequency (grey bars: percentage of males responding with male courtship song (MCrS) and male calling song (MCS); black bars: percentage of males responding with MCS only). (b) MCrS (left) and MCS (right) signalling activity of males exposed to stimuli of variable pre-pulse velocity. Reference value is indicated with the white bar.

4.2 Responsiveness of control males

In this experimental series we tested the responsiveness and quality of MCrS responses of the control males, which were immobilized and treated with 1 µl of

distilled water (N=20). No changes in mobility of insects and no mortality were recorded after the control treatment at any observed time intervals of 1, 2, 3, 24, 48 and 72 hours (h). Afterwards, control males were exposed to the stimulation program of FCS and their responses were observed within 72 h test period. Fifteen males were tested for each pre-treatment and corresponding post-treatment trial. No differences were observed between untreated and the control males in any of the tested parameters. General responsiveness of control males is shown in the Figure 17 and details on analysed parameter values are shown in Tables 8-12 (Fisher's exact test, $P<0.05$; paired, two tailed T-test, $P<0.05$). Results showed that immobilizing has no impact on physical condition and responsiveness of males to the FCS stimulation.

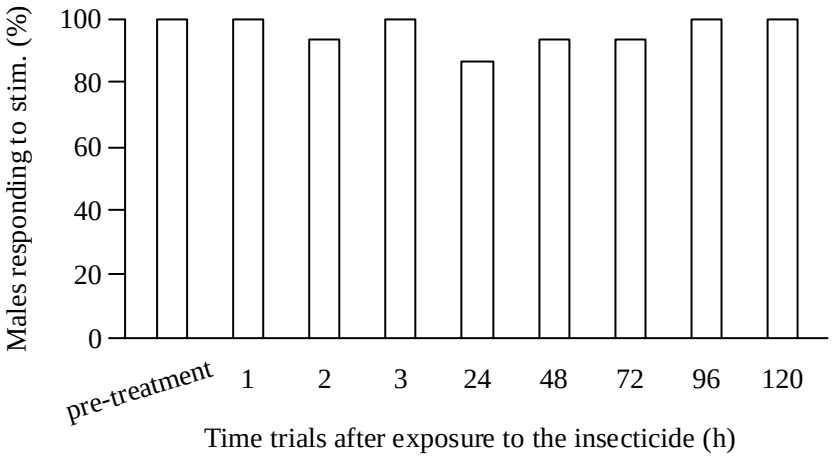

Figure 17: General responsiveness of control *Nezara viridula* males (treated with 1 µl of distilled water). Percentage of males responding to stimulation in the pre-treatment and post-treatment trials at time intervals of 1, 2, 3, 24, 48 and 72h after exposure. The percentage of males was compared between trials using Fisher's exact test ($P<0.05$). For each pre-treatment and corresponding post-treatment trial 15 males were tested.

Table 8: Duration of male courtship song (MCrS) pulse trains emitted before and after control treatment (1 µl of distilled water). Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Duration of MCrS pulse trains was compared between pre-treatment and post-treatment trials (paired T-test, P<0.05). Number of tested males was 15.

	Mean (ms)	SD	P value (paired T- test)	CV
Pre-treatment	2433	398		0.164
1 h	2441	356	0.947	0.146
Pre-treatment	2506	387		0.154
2 h	2530	452	0.865	0.179
Pre-treatment	2852	884		0.311
3 h	2913	943	0.845	0.323
Pre-treatment	2209	539		0.244
24 h	2500	493	0.102	0.197
Pre-treatment	2515	479		0.191
48 h	2810	438	0.220	0.156
Pre-treatment	2355	635		0.269
72 h	2739	557	0.167	0.203

Table 9: Number of male courtship song (MCrS) pulse trains emitted before and after control treatment (1 µl of distilled water). Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Number of MCrS pulse trains was compared between pre-treatment and post-treatment trials (paired T-test, P<0.05). Number of tested males was 15.

	Mean	SD	P value (paired T- test)	CV
Pre-treatment	15.6	5.8		0.375
1 h	15.1	6.5	0.238	0.428
Pre-treatment	16.6	5.1		0.309
2 h	14.3	4.7	0.366	0.333
Pre-treatment	12.5	10.0		0.802
3 h	7.9	5.3	0.200	0.675
Pre-treatment	13.0	4.3		0.334
24 h	13.0	4.7	1.000	0.367
Pre-treatment	6.3	4.1		0.649
48 h	5.0	3.1	0.309	0.614
Pre-treatment	5.7	4.1		0.721
72 h	6.7	3.4	0.562	0.512

Table 10: Response latency 1 (number of the FCS pulse trains needed to elicit male response) before and after control treatment (1 µl of distilled water). Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 1 was compared between pre-treatment and post-treatment trials (paired T-test, P<0.05). Number of tested males was 15.

	Mean	SD	P value (paired T- test)	CV
Pre-treatment	5.6	6.8		>1.000
1 h	2.9	1.8	0.181	0.642
Pre-treatment	7.3	5.4		0.742
2 h	7.0	7.0	0.785	0.988
Pre-treatment	6.8	5.0		0.733
3 h	8.1	4.7	0.511	0.578
Pre-treatment	6.2	5.2		0.555
24 h	7.5	4.9	0.599	0.673
Pre-treatment	7.1	8.4		0.483
48 h	12.1	8.7	0.165	0.719
Pre-treatment	7.9	2.6		0.329
72 h	10.0	6.2	0.345	0.623

Table 11: Response latency 2 (time between onset of FCS pulse train and subsequent MCrS pulse train) before and after control treatment (1 µl of distilled water). Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 2 was compared between pre-treatment and post-treatment trials (paired T-test, P<0.05). Number of tested males was 15.

	Mean (ms)	SD	P value (paired T- test)	CV
Pre-treatment	920	193		0.209
1 h	1000	168	0.207	0.168
Pre-treatment	906	108		0.119
2 h	1000	124	0.128	0.124
Pre-treatment	1045	142		0.136
3 h	1199	301	0.123	0.251
Pre-treatment	1012	111		0.109
24 h	1102	177	0.137	0.161
Pre-treatment	993	239		0.241
48 h	1142	129	0.347	0.277
Pre-treatment	1056	246		0.232
72 h	1133	213	0.468	0.188

Table 12: Dominant frequency of male courtship song (MCrS) pulse trains emitted before and after control treatment (1 µl of distilled water). Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Dominant frequency of MCrS pulse trains was compared between pre-treatment and post-treatment trials (paired T-test, P<0.05). Number of tested males was 15.

	Mean (Hz)	SD	P value (paired T- test)	CV
Pre-treatment	88.7	4.8		0.054
1 h	89.8	4.0	0.123	0.044
Pre-treatment	88.7	4.8		0.054
2 h	89.5	4.4	0.599	0.049
Pre-treatment	96.1	2.7		0.028
3 h	97.1	7.4	0.711	0.076
Pre-treatment	101.0	4.3		0.043
24 h	96.5	6.9	0.254	0.072
Pre-treatment	94.9	2.7		0.029
48 h	98.9	6.9	0.329	0.069
Pre-treatment	94.9	2.7		0.029
72 h	101.3	7.6	0.196	0.076

4.3 Toxicity of imidacloprid and deltamethrin to *N. viridula* males

In this experimental series, we determined sublethal doses of imidacloprid and deltamethrin insecticides, which were subsequently used in behavioural experiments (Table 13).

Table 13: Imidacloprid and deltamethrin doses tested for toxicity to *Nezara viridula* males. (*) sublethal doses were subsequently used in the behavioural experiments. (N) number of tested males was between 17–20 for the specific dose.

	Active ingredient (Commercial insecticide)	Active ingredient (Commercial insecticide)
	Imidacloprid (Confidor® 200 SC)	Deltamethrin (Decis® 2.5 EC)
Applied doses (ng of a.i./µl)	300.0 (N=20)	12.50 (N=20)
	150.0* (N=20)	6.25 (N=18)
	75.0* (N=17)	1.50* (N=20)
	38.5* (N=20)	
	18.5* (N=20)	

Results showed that deltamethrin is more toxic to *N. virdula* males than imidacloprid. Deltamethrin caused higher mortality than imidacloprid. LD_{50} (contact exposure, after 24 h) for deltamethrin (Decis 2.5 EC) was 12.5 ng/bug

(corresponding to the recommended field dose) and 300 ng/µl for imidacloprid (Confidor 200 SC) (two times higher from recommended field dose).

4.3.1 Imidacloprid

Table 14 summarises the percentage of affected bugs and mortality rate at different time intervals (1 and 72 h after treatment) of control males (treated with 1µl of distilled water) and of those treated with different doses of imidacloprid. Compared with the control, mortality of males was significantly higher only after imidacloprid treatment at the dose of 300 ng/µl (Fisher's exact test). First poisoning symptoms (knock-down effect, tremor, and uncoordinated movement) were observed 1 h after treatment, with 20% (4/20) of affected males. Within 72 h no recovery was observed, percentage of affected bugs decreased on the account of increased mortality. Four out of 20 treated males died after 3 h, but compared to the control, the difference was not statistically significant (Fisher's exact test, $P=0.119$). Mortality of treated males (50%, 10/20) was significantly higher from that of the control (0/20) 24 h after treatment (Fisher's exact test, $P<0.001$).

Imidacloprid treatment at lower doses (150, 75, 38.5 and 18.5 ng/µl) did not result in increased mortality; no dead bugs were recorded within 72 h test period (Table 14). Imidacloprid doses between 150 and 18.5 ng/µl were therefore considered to be sublethal and poisoning symptoms were exhibited in relatively small percentage. The highest percentage of affected males was recorded in males treated with 150 ng/µl. Partial paralysis of legs and tremor was observed in 10% (2/20) of males, within 2 and 24 h after treatment. In groups treated with 75, 38.5 and 18.5 ng/µl, five percent (1/20) of affected males was recorded and occurrence of poisoning symptoms was not time-dependent. Compared to the control, at imidacloprid doses of 150, 75, 38.5 and 18.5 ng/µl, no significant differences were recorded in physical condition and mortality at any time interval after treatment (Fisher's exact test, $P>0.05$ for all comparisons). Therefore, these four doses of imidacloprid were used in subsequent behavioural experiments (Table 13). We assessed the dose-dependent effects of the imidacloprid on the selected process of vibrational communication.

Table 14: Percentage of affected and dead *Nezara viridula* males in the control (treated with 1 µl of distilled water) and imidacloprid treated groups (at doses of 300, 150, 75, 38.5 and 18.5 ng/µl) at different time intervals after treatment (1 to 72 h). No mortalities were observed after imidacloprid treatment at doses of 150-18.5 ng/µl, therefore given is only the percentage of affected bugs. Significant difference in percentage of affected and dead males between control and treated males is indicated with asterisks (Fisher's exact test, *P<0.05, **P<0.01, ***P<0.001). Twenty males were tested for each dose.

	Control		300 ng/µl		150 ng/µl	75 ng/µl	38.5 ng/µl	18.5 ng/µl
	affected (%)	dead (%)	affected (%)	dead (%)	affected (%)	affected (%)	affected (%)	affected (%)
1 h	0	0	20	0	5	5	0	0
2 h	0	0	60***	0	10	0	0	5
3 h	0	0	80***	20	10	0	0	0
24 h	0	0	40*	50***	10	0	0	0
48 h	0	0	40*	60***	0	0	5	0
72 h	0	0	40*	60***	0	5	0	5

4.3.2 Deltamethrin

Another set of preliminary experiments was conducted to determine sublethal dose of deltamethrin (Figure 18). Compared to the control, significantly higher percent (95%, 19/20, Figure 18a) of affected males (tremor, paralysis, uncoordinated and very slow movement) was observed 1 h after exposure to deltamethrin at the initial tested dose (i.e. 12.5 ng/, corresponds to recommended field dose) (Fisher's exact test, P<0.001). No recovery was recorded and 72 h after the treatment all males died (100% mortality, 20/20). Deltamethrin treatment at the dose of 6.25 ng/µl, induced significantly lower mortality rate from that of the 12.5 ng/µl treatment (Figure 18b) at any observed time intervals after the application (Fisher's exact test, P<0.001). However, the effects of the 6.25 ng treatment were still significantly higher from that of the control: after 72 hours 50% (10/20) mortality was recorded (Fisher's exact test, P<0.001) and the percentage of affected males ranged between 10 and 50% (Figure 18b). When males were treated with the dose of 1.5 ng of deltamethrin/µl no dead bugs and no other poisoning symptoms were observed within 72 h test period. Therefore, we considered deltamethrin dose of 1.5 ng/µl as sublethal and we used this dose in the subsequent behavioural experiments.

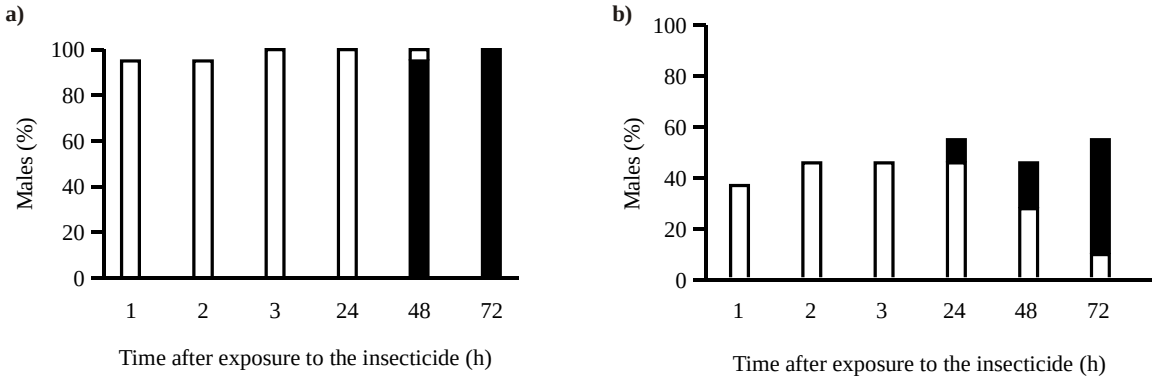

Figure 18: Percentage of affected (white circle) and dead *Nezara viridula* males (black circle) after exposure to deltamethrin at different doses. (a) 12.5 ng/µl. (b) 6.25 ng/µl. Records were made at time intervals of 1, 2, 3, 24, 48 and 72 h after exposure to the insecticide. A group of 20 males was used in the test.

4.4 Experiment 2-Effects of insecticides on male responsiveness to the female calling song (FCS)

4.4.1 Imidacloprid treatment at the dose of 150 ng/µl

General responsiveness

Figure 19 shows general responsiveness of males (percentage of males responding to synthesised FCS stimulation) before (pre-treatment trial) and after the imidacloprid treatment (post-treatment trial) at the dose of 150 ng/µl within 120 hours test period. General responsiveness of treated males was significantly lower at all time intervals after treatment. In the pre-treatment trials males exhibited 100% responsiveness, whereas 1 h after treatment, the responsiveness of males, compared to the pre-treatment, significantly decreased to 54% (Fisher's exact test, two tailed, $P<0.05$). At the time interval of 2 h, responsiveness dropped below 50%. At later time intervals (between 3 and 48 h) the level of responsiveness did not change. A decrease in responsiveness was exhibited again 72 h after treatment and maximum effect was observed after 96 h when only 21.1% (4/19) of males responded. Furthermore, after 96 hours one out of 20 treated males died. Five days (120 h) after treatment the responsiveness level increased to 57.9% (11/19), but compared to the pre-treatment trial (100% responsiveness) the percentage of responding males was still

significantly lower (Fisher's exact test, P<0.01). We can conclude that within 120 h test period, males were not able to recover completely from the effects of imidacloprid treatment at the dose of 150 ng/µl.

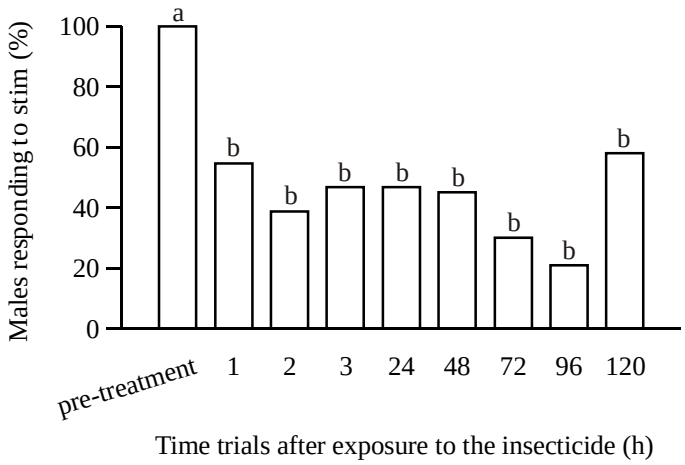

Figure 19: General responsiveness of *Nezara viridula* males treated with imidacloprid at the dose of 150 ng/µl. Percentage of males responding to stimulation in the pre-treatment and post-treatment trials at time intervals of 1, 2, 3, 24, 48 and 72h after treatment. The percentage of males was compared between trials using Fisher's exact test (P<0.05). Different letters indicate statistically different response levels. Different number of males was used in different groups of pairs of pre- and post-treatment trials: 1 h trial N=11, 2 h N=13, 3 h N=15, 24 h N=15, 48h N=20, 96 h N= 20 and 120 h trial N=20.

Relatively few males responded to stimulation after imidacloprid treatment at the dose of 150 ng/µl (between two and six, Table 15–18). We analysed the quality of their responses before (pre-treatment) and at different time intervals after treatment (post-treatment trials of 1, 2, 3 h, 24, 48 and 72 h). Figure 20 shows values of the specific MCrS parameters emitted during pre-treatment and post-treatment trails at different time intervals after treatment. Imidacloprid treatment at the dose of 150 ng/µl significantly affected the duration of the MCrS pulse trains: a significant decrease of MCrS pulse train duration were observed in five post-treatment trials (1, 2 h, 24, 48 and 72 h). Mean values of other parameters did not differ a lot (see below) between pre-treatment and post-treatment trials. Males emitted MCrS signals only during stimulation, therefore we were not able to analyse spectral characteristics of the signals that overlapped with FCS signals.

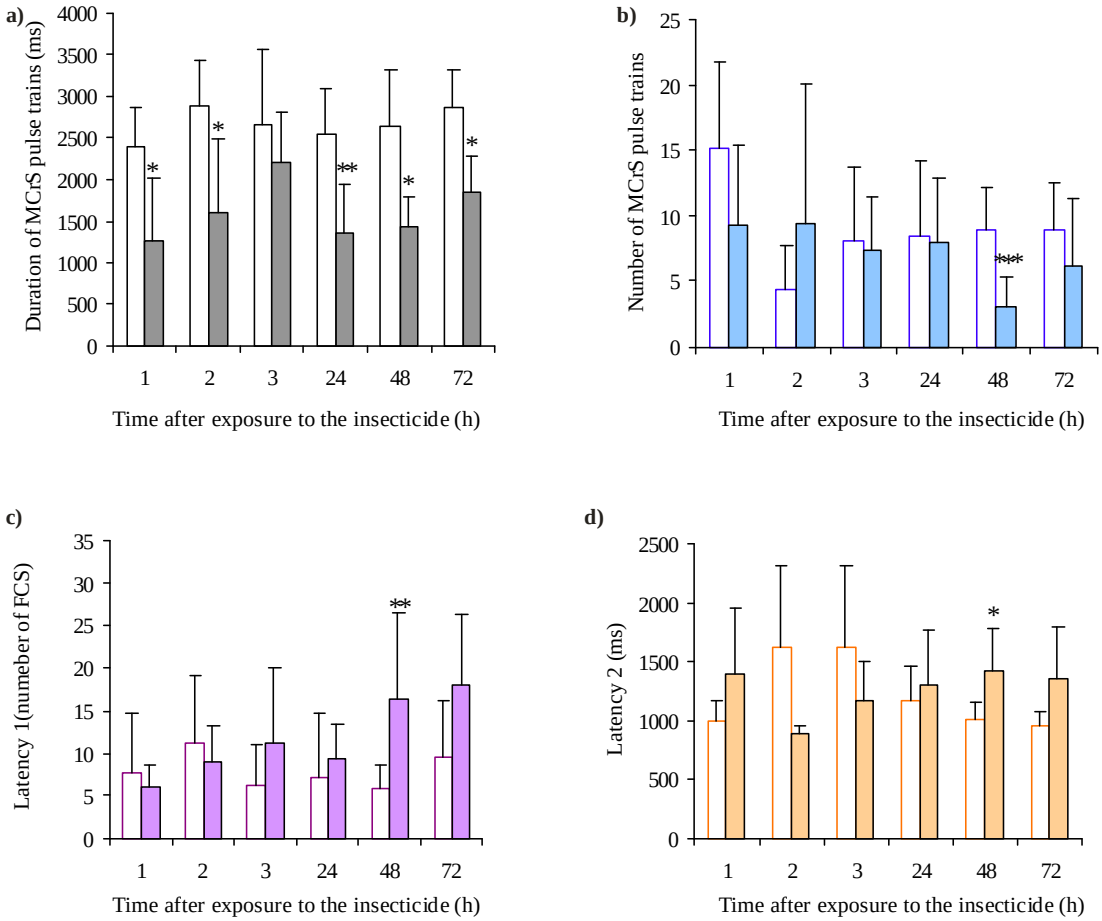

Figure 20: Quality of the *Nezara viridula* male courtship song (MCrS) response before and after imidacloprid treatment at the dose of 150 ng/µl. Shown are pre-treatment (blank bars) and corresponding post-treatment trials (solid bars) of 1, 2, 3, 24, 48 and 72 h. Bars give the mean values (±SD) of (a) MCrS pulse train duration, (b) number of emitted MCrS pulse trains, (c) response latency 1, (d) response latency 2. Asterisks indicate significant difference in mean parameter values between pre-treatment and post-treatment trials (paired T-test, *P<0.05; **P<0.01; ***P<0.001). Further details on parameter values and significance levels are given in Table 15–18.

Duration of the male courtship song (MCrS) pulse train (ms)

Duration of the MCrS pulse trains significantly decreased in all post-treatment trials (paired T-test, Figure 20a, Table 15). Except at time interval of 3 h, no significant difference was observed in MCrS duration during the pre-treatment and post-treatment trial (paired T-test, P=0.285, NS). Maximum difference between pre-treatment and post-treatment trials was observed 24 h after imidacloprid application (paired T-test, P<0.01, Table 15). More variation in MCrS pulse train duration

occurred in post-treatment trials: coefficient of variation (CV) ranged from 0.234 to 0.703 in post-treatment and from 0.161 to 0.332 in pre-treatment trials.

Table 15: Duration of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 150 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Duration of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	2394	477		0.199
1 h (N=6)	**1256**	745	**0.013**	0.467
Pre-treatment	2884	535		0.186
2 h (N=2)	**1596**	884	**0.026**	0.703
Pre-treatment	2668	888		0.332
3 h (N= 6)	2217	591	0.285 NS	0.267
Pre-treatment	2539	549		0.216
24 h (N=5)	**1361**	576	**0.002**	0.424
Pre-treatment	2651	665		0.251
48 h (N=6)	**1424**	365	**0.046**	0.234
Pre-treatment	2871	462		0.161
72 h (N=4)	**1857**	442	**0.039**	0.238

Number of MCrS pulse trains

Variation of emitted number of MCrS pulse trains was high in pre-treatment as well as in post-treatment trials (CV ranged from 0.351 to 1.000). Number of MCrS pulse trains significantly decreased only at 48 h time interval (pre-treatment vs. post-treatment: paired T-test, P<0.001, Figure 20b, Table 16). During the 2 h post-treatment trial, the mean number of emitted pulse trains (9.5) did not significantly differ from those of the pre-treatment (paired, T-test, P=0.623 NS), however in post-treatment trial one out of two responding males emitted two MCrS pulse trains and the other 17.

Table 16: Number of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 150 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Number of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	15.1	6.6		0.434
1 h (N=6)	9.4	6.1	0.106 NS	0.645
Pre-treatment	4.4	3.3		0.733
2 h (N=2)	9.5	10.6	0.623 NS	>1.000
Pre-treatment	8.0	5.9		0.702
3 h (N=6)	7.3	4.1	0.779 NS	0.563
Pre-treatment	8.4	5.8		0.689
24 h (N=5)	8.0	4.9	0.890 NS	0.612
Pre-treatment	9.0	3.2		0.351
48 h (N=6)	**3.0**	2.3	**<0.001**	0.760
Pre-treatment	9.0	3.7		0.395
72 h (N=4)	6.3	5.1	0.538 NS	0.819

Response latency 1 (number of FCS pulse trains needed to elicit male response)

The number of FCS pulse trains needed to elicit male response (response latency 1) was different in different pre-treatment and post-treatment trials (Table 17, Figure 20c). Response latency decreased one and 2 h after imidacloprid treatment at dose of 150 ng/µl, but, compared to pre-treatment trials, the difference was not significant (pre-treatment vs. post-treatment trial: paired T-test, NS, P=0.779, P=0.705, respectively). On the other hand, compared to pre-treatment an increase in response latency was recorded between 3 and 73 h after exposure to the insecticide. However, the difference was statistically significant only at the time interval of 48 h (paired T-test, Table 17). High variation in response latency 1 was characteristic for all pre-treatment and post-treatment trials (CV>0.353).

Table 17: Response latency 1 (number of FCS pulse trains needed to elicit male response) before and after imidacloprid treatment at the dose of 150 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 1 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	7.9	6.9		0.883
1 h (N=6)	6.0	2.5	0.779 NS	0.425
Pre-treatment	11.2	7.8		0.696
2 h (N=2)	9.0	4.2	0.705 NS	0.471
Pre-treatment	10.0	7.7		0.775
3 h (N=6)	12.2	4.4	0.537 NS	0.358
Pre-treatment	7.1	7.7		>1.000
24 h (N=5)	9.3	4.0	0.640 NS	0.433
Pre-treatment	5.8	2.8		0.478
48 h (N=6)	**16.3**	10.1	**0.004**	0.617
Pre-treatment	9.6	6.5		0.681
72 h (N=4)	18.0	8.2	0.163 NS	0.353

Response latency 2 (time between onset of the FCS pulse train and subsequent MCrS response; ms)

Table 18 and Figure Figure 20d show the mean values of response latency 2 in pre-treatment and post-treatment trials. Compared to pre-treatment, significantly longer response latency 2 was observed in 48 h post-treatment trial (paired, T-test, P<0.05).

Table 18: Response latency 2 (time between onset of FCS pulse train and subsequent MCrS pulse train) before and after imidacloprid treatment at the dose of 150 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 2 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	1001	168		0.168
1 h (N=6)	1385	561	0.174 NS	0.405
Pre-treatment	1621	701		0.432
2 h (N=2)	882	74,	0.118 NS	0.84
Pre-treatment	1621	701		0.432
3 h (N=6)	1167	336	0.559 NS	0.288
Pre-treatment	1171	295		0.250
24 h (N=5)	1305	463	0.661 NS	0.355
Pre-treatment	1014	139		0.137
48 h (N=6)	**1424**	365	**0.043**	0.256
Pre-treatment	961	117		0.121
72 h (N=4)	1353	443	0.163 NS	0.327

4.4.2 Imidacloprid treatment at the dose of 75 ng/µl

General responsiveness

Imidacloprid treatment at the dose of 75 ng/µl significantly decreased the general responsiveness in all, time-dependent, post-treatment trials (Figure 21). General responsiveness decreased from 100% (20/20, pre-treatment trial) to 57.8% (11/19) 1 h after treatment (Fisher's exact test, $P<0.01$) and the level of responsiveness did not change within first three hours. One out of 20 treated males showed poisoning symptoms (tremor, motionless) and could not be tested. A second decrease in responsiveness (31.25%, 5/16) was observed 24 h after treatment but compared to 1–3 h trials it was not significantly lower (Fisher's exact test, $P=0.228$, NS). At the time interval of 48 h we observed an increase in responsiveness, but compared to the pre-treatment trial the responsiveness was still significantly lower (Fisher's exact test, $P<0.001$). Within 72 h after treatment, we observed partial recovery of the male responsiveness (52.6%, 10/19), but compared to pre-treatment trial still significantly lower percentage of males responded to the stimulation (Fisher's exact test, $P<0.001$).

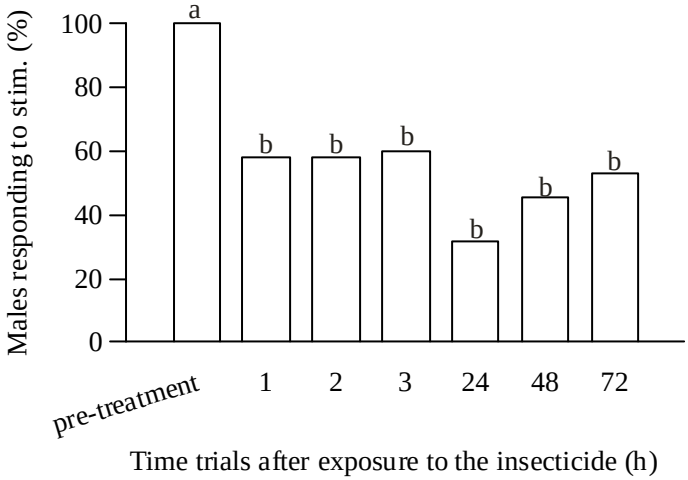

Figure 21: General responsiveness of *Nezara viridula* males treated with imidacloprid at the dose of 75 ng/µl. Percentage of males responding to stimulation in the pre-treatment and post-treatment trials at time intervals of 1, 2, 3, 24, 48 and 72h after exposure. The percentage of males was compared between trials using Fisher's exact test ($P<0.05$). Different letters indicate statistically different response levels. For each trial 20 males (N=20) were used, except for 24 h time interval N=16.

Imidacloprid at the dose of 75 ng/µl induced significant decrease in general responsiveness, in contrast, quality of the MCrS response was not affected markedly (Figure 22, Table 19–23).

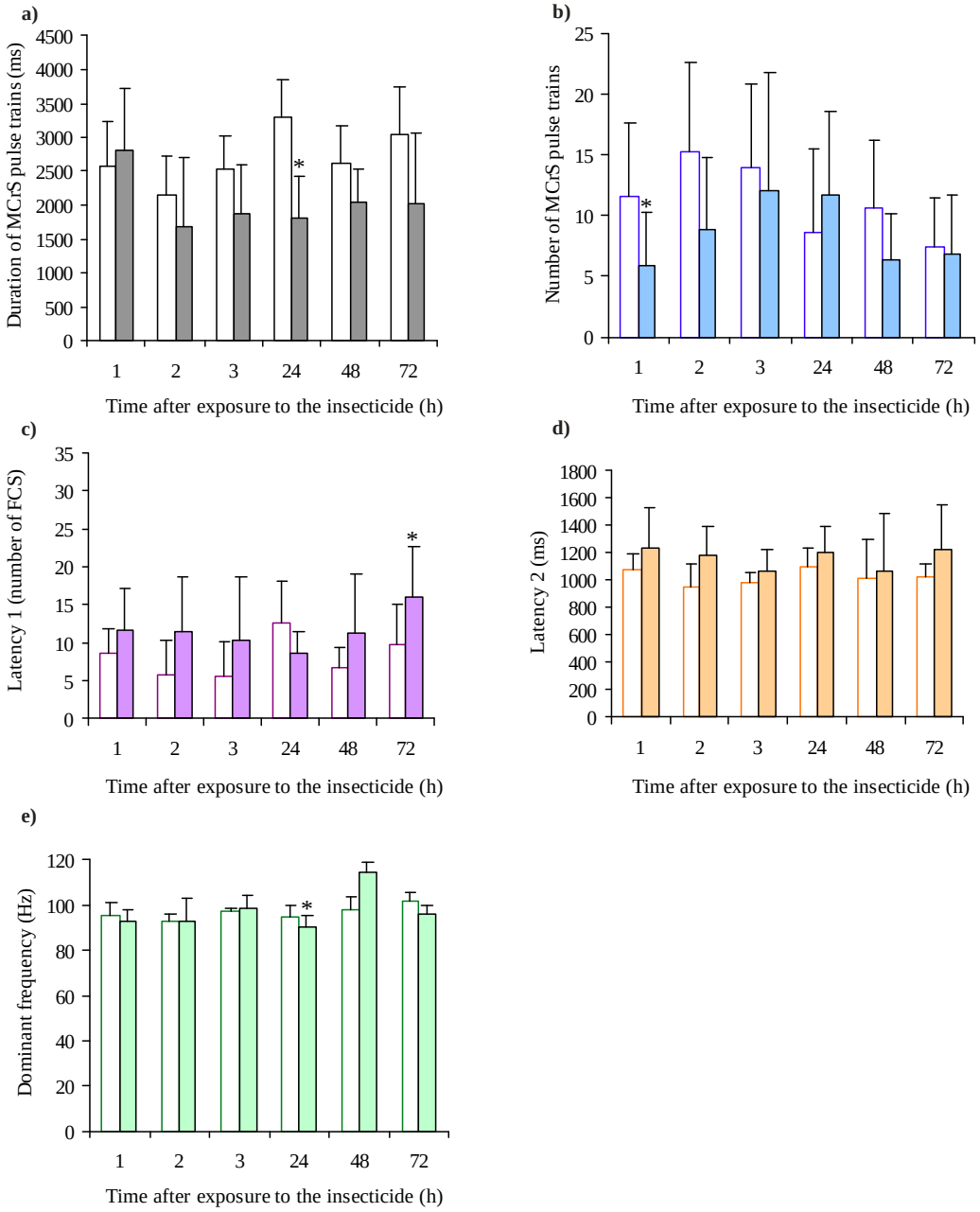

Figure 22: Quality of the *Nezara viridula* male courtship song (MCrS) response before and after imidacloprid treatment at the dose of 75 ng/µl. Shown are pre-treatment (blank bars) and corresponding post-treatment trials (solid bars) of 1, 2, 3, 24, 48 and 72 h. Bars give the mean values (±SD) of (a) MCrS pulse train duration, (b) number of emitted MCrS pulse trains, (c) response latency 1, (d) response latency 2, e) dominant frequency. Asterisks indicate significant difference in mean parameter values between pre-treatment and post-treatment trials (paired T-test, *P<0.05; **P<0.01; ***P<0.001). Further details on parameter values and significance levels are given in Table 19–23.

Duration of the MCrS pulse train (ms)

Imidacloprid treatment at the dose of 75 ng/µl induced a decrease in MCrS pulse trains duration in all post-treatment trials (except at 1 h time trial). However, compared to pre-treatment trial, the difference was statistically significant only 24 h after treatment (paired T-test, $P<0.05$, Figure 22a, Table 19). Variation of pulse train duration was higher in post-treatment trials (for pre-treatment: CV between 0.117 and 0.293; for post-treatment trials: CV between 0.243 and 0.603; Table 19).

Table 19: Duration of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 75 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Duration of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	2394	476		0.254
1 h (N=10)	1256	745	0.498 NS	0.328
Pre-treatment	2884	535		0.266
2 h (N=9)	1596	883	0.099 NS	0.603
Pre-treatment	2667	888		0.243
3 h (N=5)	2217	591	0.438 NS	0.448
Pre-treatment	2539	549		0.165
24 h (N=5)	**1361**	576	**0.043**	0.335
Pre-treatment	2651	666		0.218
48 h (N=7)	1423	365	0.077 NS	0.243
Pre-treatment	2871	462		0.117
72 h (N=7)	1857	442	0.091 NS	0.293

Number of MCrS pulse trains

In all tested time-dependant post-treatment trials (except for the 24 h one) males, treated with 75 ng of imidacloprid/µl, emitted less MCrS pulse trains than in pre-treatment trials (Figure 22b, Table 20). However, the difference was significant only 1 h after treatment (paired, two-tailed T-test, $P<0.05$). High variation in number of emitted MCrS pulse trains was observed in all trials ($CV>0.54$ in pre-treatment and post-treatment trials).

Table 20: Number of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 75 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Number of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	15.1	6.6		0.549
1 h (N=10)	**9.4**	6.1	**0.049**	0.697
Pre-treatment	4.5	3.3		0.493
2 h (N=9)	9.5	10.6	0.098 NS	0.615
Pre-treatment	8.1	5.7		0.489
3 h (N=5)	7.3	4.1	0.625 NS	0.810
Pre-treatment	8.4	5.8		0.795
24 h (N=5)	8.0	4.9	0.529 NS	0.579
Pre-treatment	9.0	3.2		0.540
48 h (N=7)	3.0	2.3	0.219 NS	0.581
Pre-treatment	9.0	3.6		0.549
72 h (N=7)	6.3	5.1	0.842 NS	0.709

Response latency 1 (number of FCS pulse trains needed to elicit the male response)

Figure 22c shows mean values of response latency 1 during pre-treatment and post-treatment trials within 72 h after exposure to imidacloprid at the dose of 75 ng/ µl. Compared to the pre-treatment, response latency 1 was higher in all post-treatment trials (except at 24 h trial), but the significant increase of the tested parameter value was observed only 72 h after exposure to imidacloprid (paired, two-tailed T-test P<0.05; Table 21, Figure 22c). High variability of the response latency 1 was observed at all treatment (CV ranged from 0.40 to 0.90).

Table 21: Response latency 1 (number of FCS pulse trains needed to elicit male response) before and after imidacloprid treatment at the dose of 75 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 1 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	8.7	6.9		0.522
1 h (N=10)	11.6	2.5	0.186 NS	0.483
Pre-treatment	5.8	7.8		0.893
2 h (N=9)	10.5	4.2	0.098 NS	0.625
Pre-treatment	5.7	7.7		0.502
3 h (N=5)	10.5	4.4	0.074 NS	0.763
Pre-treatment	12.6	7.7		0.451
24 h (N=5)	8.6	4.0	0.309 NS	0.314
Pre-treatment	6.7	2.8		0.407
48 h (N=7)	11.1	10.0	0.138 NS	0.709
Pre-treatment	9.7	6.5		0.541
72 h (N=7)	**16.3**	8.2	**0.049**	0.415

Response latency 2 (time between onset of the FCS pulse train and subsequent MCrS response; ms)

Figure 22d and Table 22 show mean values of response latency 2 before and after exposure to imidacloprid at the dose of 75 ng/µl. Compared to pre-treatment trials, response latency 2 was longer in all post-treatment trails, but the difference was not statistically significant in any of the tested time trials (paired T-test, Figure 22d, Table 22). Variation of the response latency was relatively low in pre-treatment and post-treatment trials (CV between 0.082 and 0.293).

Table 22: Response latency 2 (time between onset of FCS pulse train and subsequent MCrS pulse train) before and after imidacloprid treatment at the dose of 75 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 2 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	1001	168		0.107
1 h (N=10)	1385	561	0.148 NS	0.249
Pre-treatment	1621	700		0.171
2 h (N=9)	881	74	0.109 NS	0.186
Pre-treatment	1621	701		0.082
3 h (N=5)	1167	336	0.250 NS	0.136
Pre-treatment	1171	295		0.189
24 h (N=5)	1305	463	0.299 NS	0.164
Pre-treatment	1014	139		0.117
48 h (N=7)	1424	365	0.125 NS	0.293
Pre-treatment	961	117		0.095
72 h (N=7)	1353	443	0.167 NS	0.273

Dominant frequency of MCrS pulse trains (Hz)

Compared to pre-treatment trial, a significant decrease in mean value of dominant frequency was observed only 24 h after exposure to imidacloprid at the dose of 75 ng/µl (paired T-test P<0.05). Dominant frequency was analysed for the MCrS pulse trains emitted during no stimulation time (pause between two sequences of 35 pulse trains, Figure 5a) to avoid overlapping with FCS pulse trains. The inter-male variation in dominant frequency was relatively low (CV=0.013–0.139, Table 23, Figure 22e).

Table 23: Dominant frequency of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 75 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Dominant frequency of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (Hz)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	95.6	5.4		0.066
1 h (N=10)	92.5	5.5	0.287 NS	0.071
Pre-treatment	92.3	3.4		0.034
2 h (N=9)	92.8	10.7	0.844 NS	0.115
Pre-treatment	97.4	1.3		0.013
3 h (N=5)	98.7	5.7	0.712 NS	0.058
Pre-treatment	94.7	5.1		0.054
24 h (N=5)	**90.4**	4.7	**0.018**	0.052
Pre-treatment	98.1	5.9		0.061
48 h (N=7)	114.9	38.2	0.388 NS	0.332
Pre-treatment	101.8	3.6		0.126
72 h (N=7)	95.9	3.7	0.150 NS	0.139

4.4.3 Imidacloprid treatment at the dose of 38.5 ng/µl

General responsiveness

General responsiveness of males to FCS stimulation decreased after exposure to imidacloprid at the dose of 38.5 ng/µl. Maximum effect of imidacloprid on the responsiveness was recorded 48 h after exposure (Figure 23). A decrease in responsiveness (from 100% to 80.9% (17/21) was recorded 1 h after the treatment, but the difference between pre- and post-treatment trials was not statistically significant (Fisher's exact test, P=0.107, NS). Compared with the pre-treatment (100%, 20/20), responsiveness of males significantly decreased 2 h after treatment (70%, 14/20, Fisher's exact test, P<0.05). Maximum effect on the responsiveness was observed 48 h after treatment, when only one, out of 14 males (7.1%) responded to the stimulation (pre-treatment vs. post-treatment: Fisher's exact test, P<0.001). At time interval of 72 h we observed some recovery from initial poisoning and the responsiveness slightly increased to 21.4%, however, compared with the pre-treatment trial, the responsiveness was still significantly lower (Fisher's exact test, P<0.001).

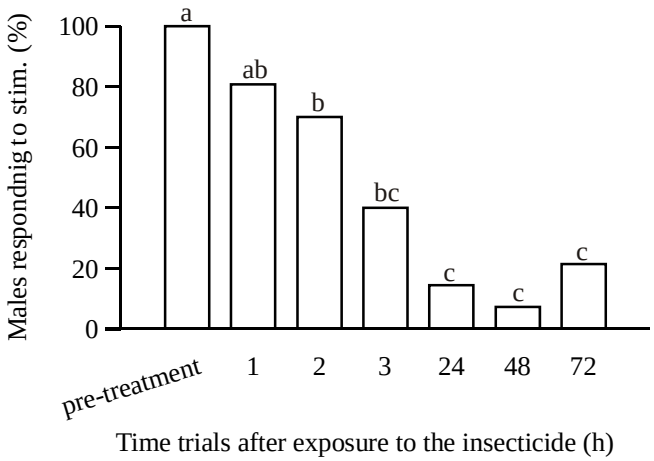

Figure 23: General responsiveness of *Nezara viridula* males treated with imidacloprid at the dose of 38.5 ng/µl. Percentage of males responding to stimulation in the pre-treatment and post-treatment trials at time intervals of 1, 2, 3, 24, 48 and 72h after exposure. The percentage of males was compared between trials using Fisher's exact test (P<0.05). Different letters indicate statistically different response levels. Twenty males were used for each time trial.

Males that responded to stimulation after imidacloprid treatment at the dose of 38.5 ng/µl, emitted pulse trains of similar characteristics than of those emitted before the treatment (Figure 24, Table 24–28). Maximum effect on the quality of the response was observed within first three after treatment and the number of MCrS pulse trains was the most affected parameter. However, 24, 48 and 72 h after treatment only two, one and three males (respectively) responded to the stimulation. Moreover, 24 h after treatment, one out of two responding males, emitted only MCS signals, the second one responded to stimulation with only one MCrS pulse train (N=1, n=1). Therefore, the effects of imidacloprid on the quality of the response were not statistically tested for 24 and 48 h time intervals.

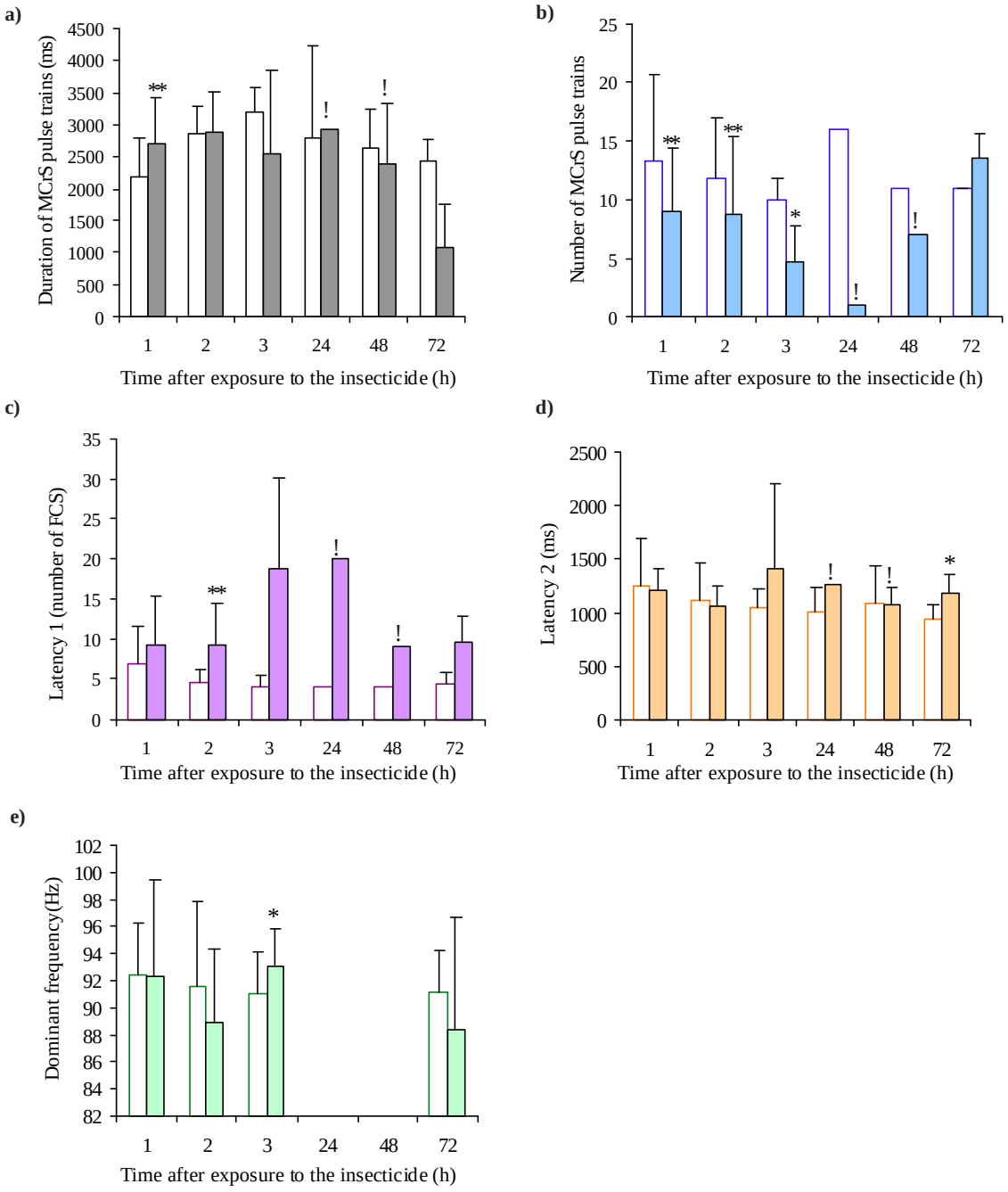

Figure 24: Quality of the *Nezara viridula* male courtship song (MCrS) response before and after imidacloprid treatment at the dose of 38.5 ng/µl. Shown are pre-treatment (blank bars) and corresponding post-treatment trials (solid bars) of 1, 2, 3, 24, 48 and 72 h. Bars give the mean values (±SD) of (a) MCrS pulse train duration, (b) number of emitted MCrS pulse trains, (c) response latency 1, (d) response latency 2, e) dominant frequency. Asterisks indicate significant difference in mean parameter values between pre-treatment and post-treatment trials (paired T-test, *P<0.05; **P<0.01; ***P<0.001). (!) For time intervals of 24 and 48 h given are data for only one responding male and the effects of imidacloprid on the quality of the response were not statistically tested for these two trials. Further details on parameter values and significance levels are given in Table 24–28.

Duration of the MCrS pulse train (ms)

Figure 24a shows differences in duration of the MCrS pulse trains before and after exposure to imidacloprid at the dose of 38.5 ng/µl within 72 h test period. Imidacloprid did not affect MCrS duration markedly, the only significant difference was observed 1 h after the treatment, when males emitted longer MCrS pulse trains than in the pre-treatment trial (paired two tailed T-test, $p<0.01$, Table 24). Data for only one responding male is given for time intervals of 24 and 48 h, therefore the effects of imidacloprid were not statistically tested for these two trials. Higher variation of duration was observed in post-treatment (CV ranging from 0.21 to 0.63) than in pre-treatment trials (CV ranging from 0.129 to 0.275).

Table 24: Duration of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 38.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Duration of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). Data for only one responding male is given for time intervals of 24 and 48 h, therefore the effects of imidacloprid were not statistically tested for these two trials.

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	2195	603		0.275
1 h (N=14)	**2716**	714	**0.009**	0.263
Pre-treatment	2874	415		0.144
2 h (N=12)	2880	633	0.968 NS	0.219
Pre-treatment	3197	388		0.121
3 h (N=4)	2555	1292	0.375 NS	0.506
Pre-treatment	2801	1438		/
24 h (N=1)	2932	/	/	/
Pre-treatment	2640	597		/
48 h (N=1)	2399	945	/	/
Pre-treatment	2436	333		0.136
72 h (N=2)	1080	689	0.117 NS	0.637

Number of MCrS pulse trains

Data on the number of MCrS pulse trains emitted before and after treatment (38.5 ng of imidacloprid/µl) are presented in Table 25 and Figure 24b. Compared to pre-treatment, the number of MCrS pulse trains significantly decreased at 1, 2 and 3 h post-treatment trails (paired T-test, Table 25). In the 24 and 48 h post-treatment trials

only one male responded (MCrS) to the stimulation and the values given represents the response of one male.

Table 25: Number of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 38.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Number of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time intervals of 24 and 48 h given are data for only one responding male and the effects of imidacloprid were not statistically tested for these two trials.

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	13.3	7.4		0.242
1 h (N=14)	**9.1**	5.2	**0.002**	0.726
Pre-treatment	11.9	5.1		0.416
2 h (N=12)	**8.8**	6.5	**0.009**	>1.000
Pre-treatment	10.0	1.8		0.332
3 h (N=4)	**4.8**	2.9	**0.024**	0.738
Pre-treatment	16.0	/		/
24 h (N=1)	1.0	/	/	/
Pre-treatment	11.0	/		/
48 h (N=1)	7.0	/	/	/
Pre-treatment	11.0	0.0		
72 h (N=2)	13.5	2.1	0.344 NS	0.170

Response latency 1 (number of FCS pulse trains needed to elicit the male response)

Response latency 1 was higher and more variable during post-treatment than pre-treatment trials. The difference between pre- and post-treatment was statistically significant only at 2 h time interval (paired T-test, P<0.01; Table 26, Figure 24c). At time interval of 24 and 48 h, only one value of one responding male is given. Before the treatment the male responded to the fourth FCS pulse train in the sequence, while 24 and 48 h after treatment 20 and 9 (respectively) FCS pulse trains were needed to elicit the male response.

Table 26: Response latency 1 (number of FCS pulse trains needed to elicit male response) before and after imidacloprid treatment at the dose of 38.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 1 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time intervals of 24 and 48 h given are data for only one responding male and the effects of imidacloprid were not statistically tested for these two trials.

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	6.9	4.8		0.698
1 h (N=14)	9.3	6.1	0.223 NS	0.659
Pre-treatment	4.5	1.7		0.385
2 h (N=12)	**9.3**	5.3	**0.009**	0.574
Pre-treatment	4.0	1.4		0.354
3 h (N=4)	18.8	11.3	0.089 NS	0.604
Pre-treatment	4.0	/		/
24 h (N=1)	20.0	/	/	/
Pre-treatment	4.0	/		/
48 h (N=1)	9.0	/	/	/
Pre-treatment	4.3	1.5		0.352
72 h (N=1)	9.7	3.2	0.189 NS	0.333

Response latency 2 (time between onset of the FCS pulse train and subsequent MCrS response; ms)

The mean values of response latency 2 (time between onset of the FCS pulse train and onset of the male response) did not markedly differ between pre- and post-treatment trials. Compared to the pre-treatment, response latency 2 was significantly longer only at the 72 h post-treatment trial (paired T-test, P<0.05, Table 27, Figure 24d).

Table 27: Response latency 2 (time between onset of FCS pulse train and subsequent MCrS pulse train) before and after imidacloprid treatment at the dose of 38.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 1 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time intervals of 24 and 48 h given are data for only one responding male and the effects of imidacloprid were not statistically tested for these two trials.

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	1255	439		0.349
1 h (N=14)	1214	195	0.719 NS	0.160
Pre-treatment	1126	342		0.304
2 h (N=12)	1069	177	0.561 NS	0.166
Pre-treatment	1057	159		0.151
3 h (N=4)	1407	797	0.504 NS	0.567
Pre-treatment	1003	229		/
24 h (N=1)	1270	/	/	/
Pre-treatment	1099	338		/
48 h (N=1)	1086	143	/	/
Pre-treatment	937	142		0.152
72 h (N=1)	**1185**	178	**0.015**	0.151

Dominant frequency of MCrS pulse trains (Hz)

Table 28 and Figure 24e show the mean values of dominant frequency of the MCrS pulse trains emitted before and after imidacloprid treatment at the dose of 38.5 ng/µl. The inter-male variation in dominant frequency was low (CV ranging from 0.044 to 0.129). Compared to the pre-treatment, the dominant frequency of emitted MCrS was significantly higher 3 h after treatment (paired T-test, P<0.05). During post-treatment trials of 24 and 48 h, one male responded to stimulation and no signals were produced during pause, therefore spectral analyses were not performed for these two trials.

Table 28: Dominant frequency of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 38.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Dominant frequency of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time intervals of 24 and 48 h dominant frequency was not analysed. Males did not respond during no stimulation period of the stimulation program.

	Mean (Hz)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	92.4	3.9		0.056
1 h (N=14)	92.3	7.1	0.977 NS	0.081
Pre-treatment	91.6	6.2		0.044
2 h (N=12)	88.9	5.5	0.052 NS	0.105
Pre-treatment	91.0	3.1		0.101
3 h (N=4)	**93.0**	2.8	**0.025**	0.057
Pre-treatment	/	/		0.050
24h (N=1)	/	/	/	0.049
Pre-treatment	/	/		0.059
48h (N=1)	/	/	/	0.099
Pre-treatment	91.1	3.1		0.112
72h (N=2)	88.4	8.3	0.729 NS	0.129

4.4.4 Imidacloprid treatment at the dose of 18.5 ng/µl

General responsiveness

Imidacloprid at the lowest tested dose (18.5 ng/µl) induced a significant decrease in general responsiveness of males to FCS stimulation in all post-treatment trials within 72 h test period (Figure 25). The first effect was exhibited 1 h after exposure to insecticide: the pre-treatment responsiveness of 100% significantly dropped to 55% during post-treatment trial (11/20, Fisher's exact test, P<0.01). One to 3 hours after exposure to insecticide males responded approximately at the same level (50%). Males responsiveness dropped to 35% (7/20) 24 h after treatment (3 h vs. 24 h, Fisher's exact test, P=0.523, NS). Partial recovery and increase in the responsiveness (57.9%, 11/19) was observed again 48 h after treatment, but compared to pre-treatment trial, significantly fewer males responded to stimulation (Fisher's exact test, P<0.001). During 72 h post-treatment trial males still exhibited significantly lower responsiveness (52.6%, 10/19) than during pre-treatment (Fisher's exact test, P<0.01). Males were not able to recover from poisoning effects of imidacloprid treatment at the dose of the 18.5 ng/µl within 72 h test period.

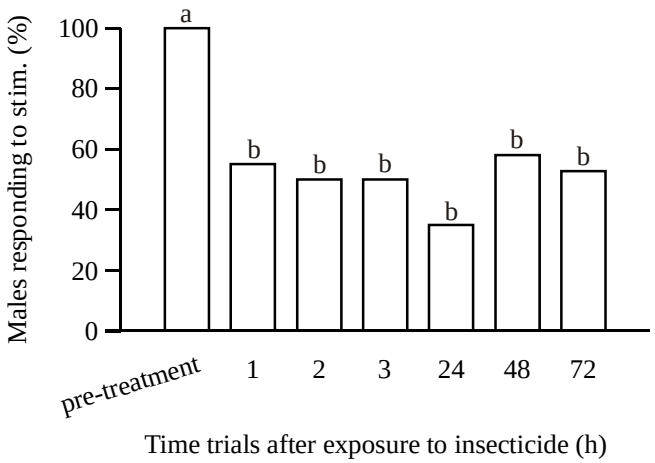

Figure 25: General responsiveness of *Nezara viridula* males treated with imidacloprid at the dose of 18.5 ng/µl. Percentage of males responding to stimulation in the pre-treatment and post-treatment trials at time intervals of 1, 2, 3, 24, 48 and 72h after exposure. The percentage of males was compared between trials using Fisher's exact test (P<0.05). Different letters indicate statistically different response levels. Twenty males were used for each time trial.

Imidacloprid treatment at the dose of 18.5 ng/µl did not markedly affect the quality of the MCrS response. Males that responded to the stimulation after exposure to insecticide generally emitted MCrS pulse trains of similar characteristics as before treatment. However, some differences were observed, mainly at the time interval of 24 h or more (Figure 26, Table 29–33).

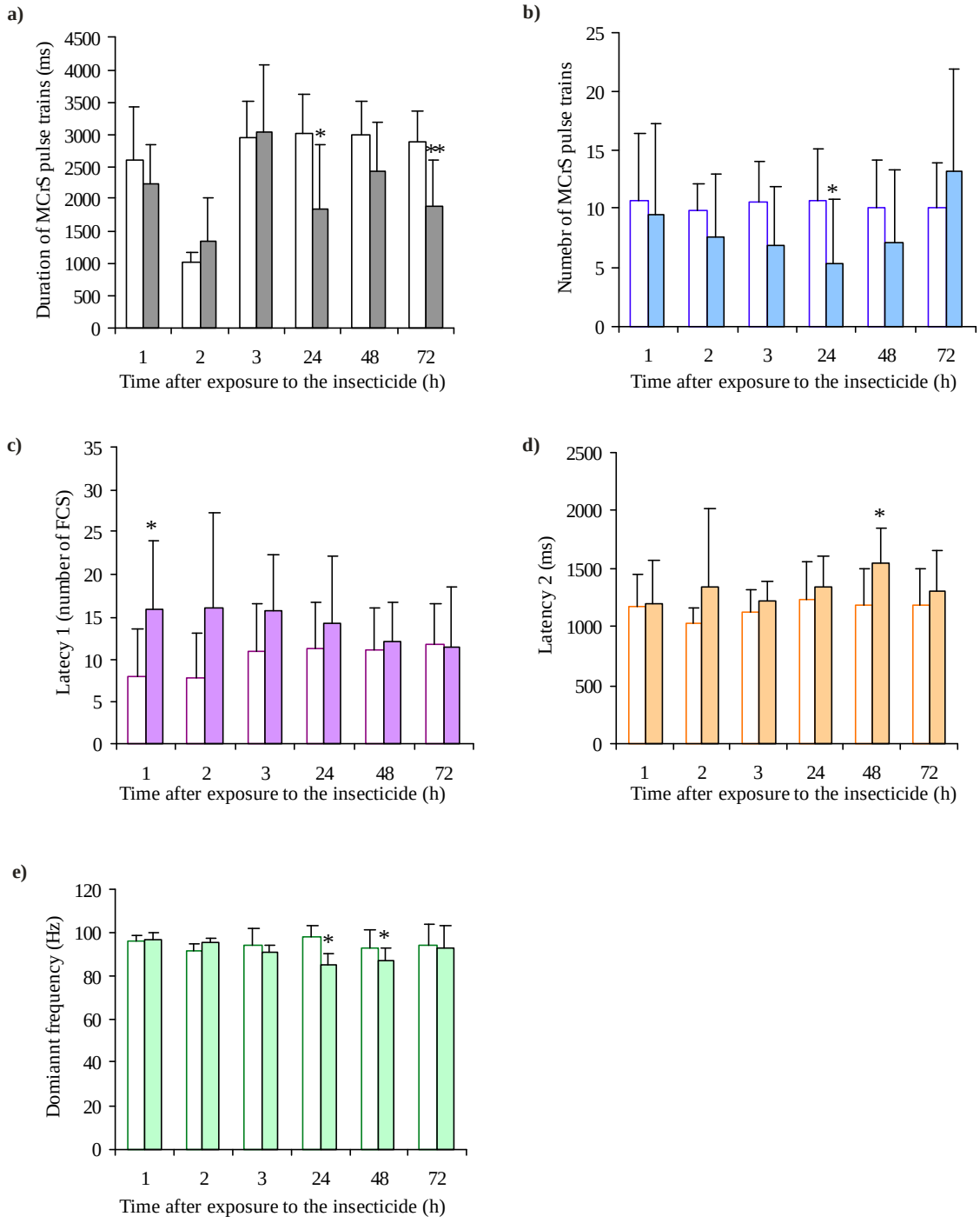

Figure 26: Quality of the *Nezara viridula* male courtship song (MCrS) response before and after imidacloprid treatment at the dose of 18.5 ng/µl. Shown are pre-treatment (blank bars) and corresponding post-treatment trials (solid bars) of 1, 2, 3, 24, 48 and 72 h. Bars give the mean values (±SD) of (a) MCrS pulse train duration, (b) number of emitted MCrS pulse trains, (c) response latency 1, (d) response latency 2, e) dominant frequency. Asterisks indicate significant difference in mean parameter values between pre-treatment and post-treatment trials (paired T-test, *P<0.05; **P<0.01; ***P<0.001). Further details on parameter values and significance levels are given in Table 29–33

Duration of the MCrS pulse train (ms)

Table 29 and Figure 26a show the mean values of MCrS pulse trains duration before and after imidacloprid treatment at the dose of 18.5 ng/µl at different time intervals within 72 h test period. Imidacloprid at the dose of 18.5 ng/µl induced a significant decrease in pulse train duration 24 and 72 h after treatment (paired T-test: $P<0.05$; $P<0.01$, respectively). The variability in duration of pulse trains was higher in post-treatment than in pre-treatment trials (CV pre-treatment ranged from 0.176 to 0.20; CV post-treatment trials ranged from 0.257 to 0.383).

Table 29: Duration of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 18.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Duration of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	2596	836		0.206
1 h (N=10)	2238	595	0.194 NS	0.257
Pre-treatment	1026	138		0.200
2 h (N=7)	1350	671	0.341 NS	0.348
Pre-treatment	2940	584		0.206
3 h (N=9)	3043	1017	0.815 NS	0.348
Pre-treatment	3020	623		0.206
24 h (N=7)	**1848**	985	**0.043**	0.533
Pre-treatment	2999	517		0.173
48 h (N=10)	2426	764	0.065 NS	0.315
Pre-treatment	2883	471		0.163
72 h (N=10)	**1885**	721	**0.004**	0.383

Number of MCrS pulse trains

Table 30 and Figure 26b represent data on the number of pulse trains emitted before and after imidacloprid treatment at the dose of 18.5 ng/µl. Compared to the pre-treatment, the number of pulse trains decreased with time in post-treatment trials. Maximum effect was recorded after 24 h, when the number of emitted pulse trains significantly decreased (paired T-test, $P<0.05$). Number of pulse trains increased again at time intervals of 48 and 72 h but it did not significantly differ from the values in the pre-treatment trials. Number of emitted pulse trains was variable in all groups of males tested, but higher variation was observed in post-treatment trials.

Table 30: Number of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 18.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Number of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	107	5.8		0.539
1 h (N=10)	9.6	7.6	0.634 NS	0.789
Pre-treatment	9.8	2.4		0.242
2 h (N=7)	7.5	5.4	0.423 NS	0.726
Pre-treatment	10.6	3.5		0.332
3 h (N=9)	6.9	5.1	0.127 NS	0.738
Pre-treatment	10.7	4.4		0.416
24 h (N=7)	**5.3**	5.5	**0.043**	>1.000
Pre-treatment	10.1	4.1		0.408
48 h (N=10)	7.2	6.1	0.157 NS	0.854
Pre-treatment	10.1	3.9		0.389
72 h (N=10)	13.2	8.7	0.200 NS	0.658

Response latency 1 (number of FCS pulse trains needed to elicit the male response)

The mean values of response latency 1 before and after imidacloprid treatment at the dose of 18.5 ng/µl is shown in Table 31 and Figure 26c. Compared to pre-treatment trial, a decrease in the response latency 1 was observed at all post-treatment trials, but the difference was statistically significant 1 h after treatment (paired T-test, P<0.05). High variation of the latency values was observed in pre-treatment as well as in post-treatment trials.

Table 31: Response latency 1 (number of FCS pulse trains needed to elicit male response) before and after imidacloprid treatment at the dose of 18.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 1 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	8.0	5.6		0.369
1 h (N=10)	**15.9**	8.1	**0.019**	0.512
Pre-treatment	7.9	5.2		0.568
2 h (N=7)	16.1	11.3	0.161 NS	0.703
Pre-treatment	10.9	5.7		0.522
3 h (N=9)	15.6	6.7	0.277 NS	0.527
Pre-treatment	11.3	5.4		0.619
24 h (N=7)	14.3	7.9	0.491 NS	0.545
Pre-treatment	11.2	4.9		0.619
48 h (N=10)	12.0	4.7	0.721 NS	0.393
Pre-treatment	11.8	4.7		0.401
72 h (N=10)	11.5	6.9	0.915 NS	0.606

Response latency 2 (time between onset of the FCS pulse train and subsequent MCrS response; ms)

The mean values of response latency 2 before and after imidacloprid treatment at the dose of 18.5 ng/µl is shown in Table 32 and Figure 26d. Compared to the pre-treatment, longer response latency 2 was recorded at all post-treatment trials, however the difference was significant only 48 h after exposure to insecticide (paired T-test, $P<0.05$).

Table 32: Response latency 2 (time between onset of FCS pulse train and subsequent MCrS pulse train) before and after imidacloprid treatment at the dose of 18.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 2 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	1185	268		0.226
1 h (N=10)	1210	360	0.741 NS	0.298
Pre-treatment	1026	138		0.135
2 h (N=7)	1350	671	0.164 NS	0.497
Pre-treatment	1126	197		0.175
3 h (N=9)	1220	172	0.208 NS	0.141
Pre-treatment	1227	337		0.275
24 h (N=7)	1345	273	0.234 NS	0.203
Pre-treatment	1195	298		0.249
48 h (N=10)	**1552**	299	**0.002**	0.193
Pre-treatment	1197	296		0.247
72 h (N=10)	1307	356	0.242 NS	0.273

Dominant frequency of MCrS pulse trains (Hz)

Compared to pre-treatment trials, no significant differences were observed in dominant frequency of the emitted MCrS pulse trains within the first two hours after imidacloprid treatment at the dose of 18.5 ng/µl (paired T-test, $P>0.05$, Table 33, Figure 26e). Dominant frequencies of MCrS pulse trains emitted during 24 and 48 h post-treatment trials were significantly lower than of those produced during the pre-treatment (paired T-test, $P<0.05$). No significant difference was observed in mean values of dominant frequencies of pulse trains between pre-treatment and post-treatment trials 72 h after exposure to insecticide (paired T-test, $P<0.05$).

Table 33: Dominant frequency of male courtship song (MCrS) pulse trains emitted before and after imidacloprid treatment at the dose of 18.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Dominant frequency of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05).

	Mean (Hz)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	96.1	2.7		0.015
1 h (N=10)	97.1	3.2	0.688 NS	0.020
Pre-treatment	91.6	3.0		0.032
2 h (N=7)	95.7	1.9	0.184 NS	0.021
Pre-treatment	94.0	7.9		0.084
3 h (N=9)	90.6	3.3	0.156 NS	0.036
Pre-treatment	97.9	5.2		0.053
24 h (N=7)	**84.9**	5.2	**0.010**	0.061
Pre-treatment	92.4	9.1		0.099
48 h (N=10)	**87.2**	5.5	**0.039**	0.063
Pre-treatment	94.2	9.7		0.103
72 h (N=10)	92.4	10.6	0.531 NS	0.115

4.4.5 Deltamethrin treatment at the dose of 1.5 ng/µl

General responsiveness

Deltamethrin treatment at the dose of 1.5ng/µl induced strong decrease in general responsiveness of males to FCS stimulation, however fast and complete recovery was observed as well (Figure 27). Compared to 100% (22/22) responsiveness during pre-treatment, the responsiveness dropped to 63.6% (14/22) 1 h after treatment, but the difference was not statistically significant (Fisher's exact test, P=0.090, NS). Compared to the pre-treatment, significantly lower performance (14.3%, 2/14) of males was recorded 2 h after treatment (Fisher's exact test, P<0.001). Male responsiveness again increased to 45% (9/20) 3 h after exposure to insecticide, but compared to pre-treatment trial (100%) the responsiveness was still significantly lower (Fisher's exact test, P<0.001). Males exhibited recovery from intoxication 48 h after treatment, when level of general responsiveness (88.9%) almost reached the level of the pre-treatment trial (100%) (Fisher's exact test, P=0.462 NS).

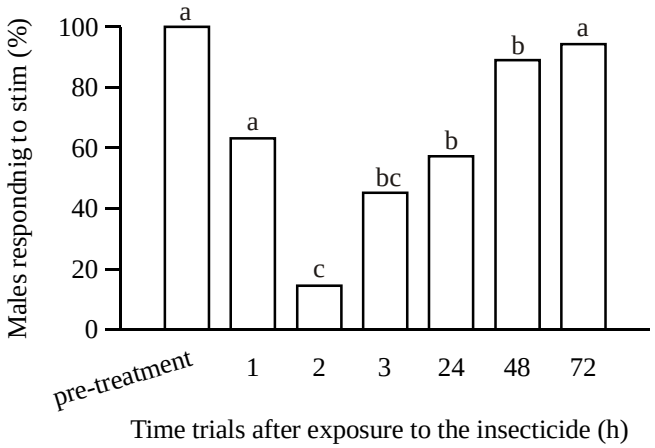

Figure 27: General responsiveness of *Nezara viridula* males treated with deltamethrin at the dose of 1.5 ng/µl. Percentage of males responding to stimulation in the pre-treatment and post-treatment trials at time intervals of 1, 2, 3, 24, 48 and 72h after exposure. The percentage of males was compared between trials using Fisher's exact test (P<0.05). Different letters indicate statistically different response levels. Twenty males were used for each time trial.

Deltamethrin treatment at the dose of 1.5 ng/µl did not induce many differences in the quality of male MCrS response (Figure 28, Table 34–38). The mean values of tested parameters did not generally differ between pre-treatment and post-treatment trials. The only significant differences were observed in the values of the MCrS pulse trains dominant frequency.

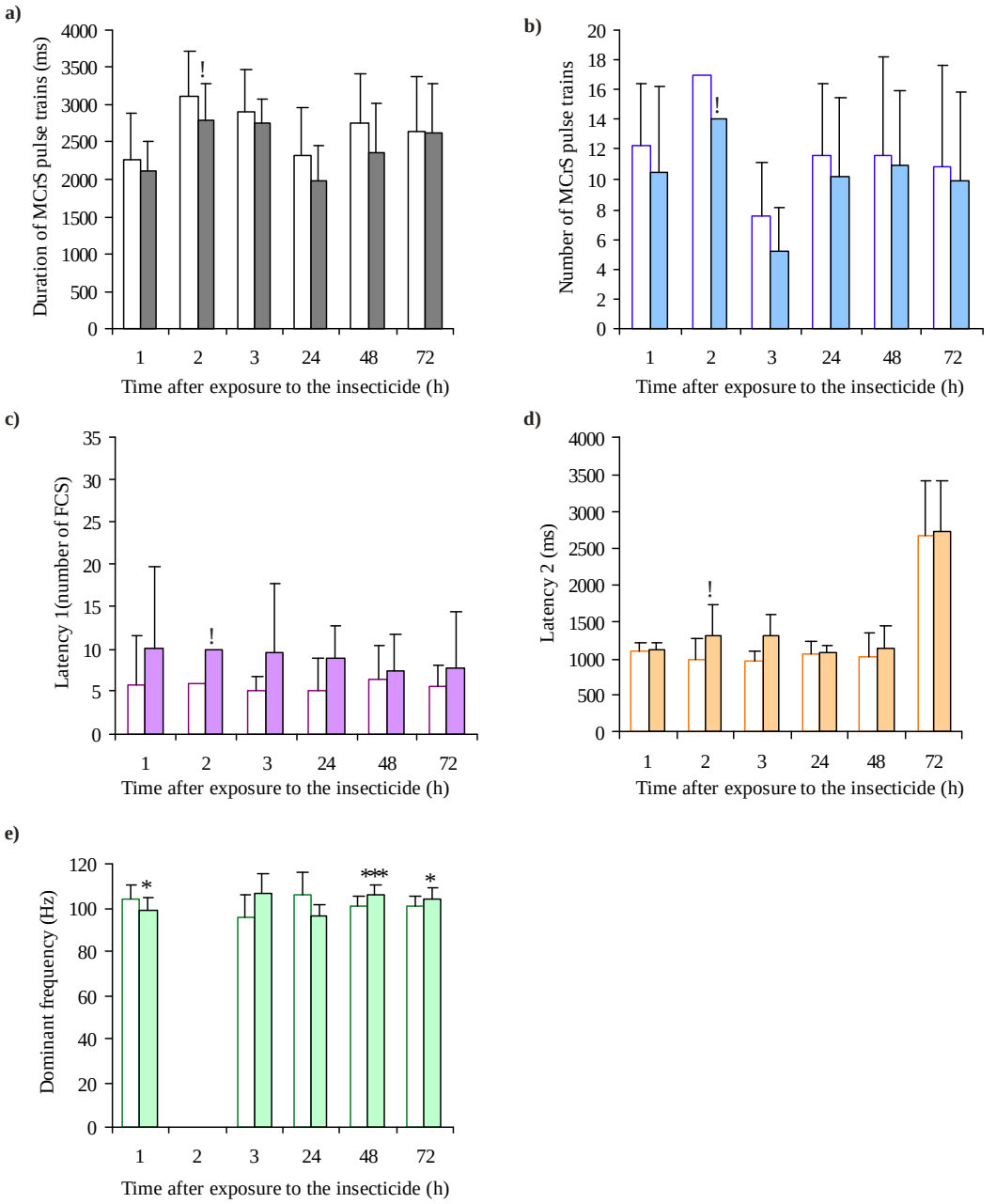

Figure 28: Quality of the *Nezara viridula* male courtship song (MCrS) response before and after exposure to deltamethrin at the dose of 1.5 ng/µl. Shown are pre-treatment (blank bars) and corresponding post-treatment trials (solid bars) of 1, 2, 3, 24, 48 and 72 h. Bars give the mean values (±SD) of (a) MCrS pulse train duration, (b) number of emitted MCrS pulse trains, (c) response latency 1, (d) response latency 2, e) dominant frequency. Asterisks indicate significant difference in mean parameter values between pre-treatment and post-treatment trials (paired T-test, *P<0.05; **P<0.01; ***P<0.001). (!) For time interval of 2 h given are data for one responding male and the effects of deltamethrin on the quality of the response were not statistically tested for this trial. Dominant frequency was not analysed for time interval of 2 h, because males did not respond during no stimulation period of the stimulation program. Further details on parameter values and significance levels are given in Table 34–38.

Duration of MCrS pulse trains (ms)

Table 34 and Figure 28a, show the mean values of duration of the MCrS pulse trains before and after deltamethrin treatment at the dose of 1.5 ng/µl within 72 h test period. Compared to the pre-treatment, males emitted shorter MCrS pulse trains in all post-treatment trials, but no significant differences were observed before and after exposure to deltamethrin any observed time intervals (paired T-test, Table 34).

Table 34: Duration of male courtship song (MCrS) pulse trains emitted before and after deltamethrin treatment at the dose of 1.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Duration of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time interval of 2 h given are data for only one responding male and the effects of deltamethrin were not statistically tested for this trial.

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	2268	625		0.392
1 h (N=5)	2108	393	0.687 NS	0.246
Pre-treatment	3096	624		/
2 h (N=1)	2794	488	/	/
Pre-treatment	2909	564		0.194
3 h (N=5)	2749	324	0.548 NS	0.118
Pre-treatment	2329	627		0.269
24 h (N=6)	1964	492	0.396 NS	0.251
Pre-treatment	2749	680		0.239
48 h (N=8)	2351	681	0.061 NS	0.289
Pre-treatment	2651	731		0.276
72 h (N=15)	2626	665	0.931 NS	0.253

Number of MCrS pulse trains

Data on the number of the MCrS pulse trains before and after deltamethrin treatment at the dose of 1.5 ng/µl are shown in Table 35 and Figure 28b. Number of emitted MCrS pulse trains decreased at all time intervals after exposure to deltamethrin, but the differences were not statistically significant (paired T-test, Table 35). Moreover, with time, the differences between pre-treatment and post-treatment trials became even smaller. High variation in the number of emitted pulse trains was observed in pre-treatment and post-treatment (CV values ranged from 0.345 to 0.632).

Table 35: Number of male courtship song (MCrS) pulse trains emitted before and after deltamethrin treatment at the dose of 1.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Number of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time interval of 2 h given are data for only one responding male and the effects of deltamethrin were not statistically tested for this trial.

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	12.2	4.2		0.345
1 h (N=59	10.4	5.8	0.121 NS	0.559
Pre-treatment	17.0	/		/
2 h (N=1)	14.0	/	/	/
Pre-treatment	7.6	3.5		0.461
3 h (N=5)	5.2	2.9	0.332 NS	0.567
Pre-treatment	11.7	4.8		0.408
24 h (N=6)	10.2	5.3	0.287 NS	0.518
Pre-treatment	11.6	6.6		0.567
48 h (N=8)	10.9	4.9	0.698 NS	0.457
Pre-treatment	10.8	6.8		0.632
72 h (N=15)	9.9	5.9	0.559 NS	0.606

Response latency 1 (number of FCS pulse trains needed to elicit the male response)

Mean values of response latency 1 before and after deltamethrin treatment at the dose of 1.5 ng/µl are shown in Table 36 and Figure 28c. Compared to the pre-treatment, an increase in response latency 1 was recorded at all post-treatment trials, but the differences were not statistically significant at any observed time intervals (paired T-test). Compared to pre-treatment, higher variation in response latency 1 was observed in post-treatment trials (Table 36).

Table 36: Response latency 1 (number of FCS pulse trains needed to elicit male response) before and after deltamethrin treatment at the dose of 1.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 1 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time interval of 2 h given are data for only one responding male and the effects of deltamethrin were not statistically tested for this trial.

	Mean	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	5.8	5.8		0.993
1 h (N=5)	10.2	9.6	0.387	0.944
Pre-treatment	6.0	/		/
2 h (N=1)	10.0	/	/	/
Pre-treatment	5.0	1.9		0.374
3 h (N=5)	9.6	8.1	0.332	0.848
Pre-treatment	5.1	3.9		0.773
24 h (N=6)	8.9	3.9	0.066	0.439
Pre-treatment	6.5	3.9		0.601
48 h (N=8)	7.3	4.5	0.569	0.619
Pre-treatment	5.6	2.4		0.421
72 h (N=15)	7.7	6.6	0.265	0.861

Response latency 2 (time between onset of the FCS pulse train and subsequent MCrS response; ms)

Deltamethrin treatment at the dose of 1.5 ng/µl did not induce significant differences in the response latency 2 (Table 37, Figure 28d, paired T-test).

Table 37: Response latency 2 (time between onset of FCS pulse train and subsequent MCrS pulse train) before and after deltamethrin treatment at the dose of 1.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Response latency 2 was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). For time interval of 2 h given are data for only one responding male and the effects of deltamethrin were not statistically tested for this trial.

	Mean (ms)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	1088	124		0.116
1 h (N=5)	1104	112	0.806 NS	0.093
Pre-treatment	998	278		/
2 h (N=1)	1307	427	/	/
Pre-treatment	972	110		0.113
3 h (N=5)	1318	275	0.073 NS	0.208
Pre-treatment	1052	171		0.163
24 h (N=6)	1061	104	0.917 NS	0.097
Pre-treatment	1037	315		0.303
48 h (N=8)	1122	309	0.431 NS	0.276
Pre-treatment	2671	749		0.302
72 h (N=15)	2733	688	0.869 NS	0.222

Dominant frequency of MCrS pulse trains (Hz)

Data on mean values of dominant frequency of the MCrS pulse trains emitted before and after exposure to deltamethrin at the dose of 1.5 ng/ µl are presented in Table 38 and Figure 28e. Compared to the pre-treatment, the dominant frequency of the MCrS pulse trains emitted during 1 h post-treatment trial significantly decreased (paired T-test, $P<0.05$). On the other hand, 48 and 72 h after treatment, significant increase of the mean frequency values was recorded (paired T-test $P<0.001$; $P<0.05$, respectively). Dominant frequency was not analysed for 2 h trial, because males did not respond during no stimulation period of the stimulation program.

Table 38: Dominant frequency of male courtship song (MCrS) pulse trains emitted before and after deltamethrin treatment at the dose of 1.5 ng/µl. Given are mean values (±SD) and coefficient of variation (CV) for pre-treatment and post-treatment trials at different time intervals (1 to 72 h). Dominant frequency of MCrS pulse trains was compared between pre-treatment and post-treatment trials, significant differences are indicated with bold letters (paired T-test, P<0.05). Dominant frequency was not analysed for 2 h time trial, because males did not respond during no stimulation period of the stimulation program.

	Mean (Hz)	SD	P value (Paired two tailed T-test)	CV
Pre-treatment	103.9	6.7		0.065
1 h (N=5)	**98.7**	5.5	**0.042**	0.055
Pre-treatment	/	/		/
2 h (N=1)	/	/	/	/
Pre-treatment	95.6	9.9		0.104
3 h (N=5)	106.2	8.8	0.051	0.820
Pre-treatment	105.8	9.9		0.093
24 h (N=6)	96.4	5.6	0.084	0.058
Pre-treatment	100.8	4.2		0.041
48 h (N=8)	**105.6**	4.9	**0.0007**	0.047
Pre-treatment	100.8	4.1		0.041
72 h (N=15)	**103.8**	5.6	**0.028**	0.054

4.4.6 Effects of deltamethrin and imidacloprid on male responsiveness to the FCS-Summary

The results of dose- and time-dependant effects of imidacloprid and deltamethrin on the vibrational communication of *N. viridula* males are summarised in Table 39. They show that general responsiveness of males to FCS stimulation decreased after insecticide treatment independently of the type and dose of the insecticide. On the

other hand, time-course of the two insecticides actions was type- and dose-dependant. Peak effect of deltamethrin (1.5 ng/µl) was exhibited 2 h after treatment, male responsiveness fully recovered and returned to the pre-treatment level 48 hours after exposure to the insecticide. Imidacloprid treatment at three tested doses (150, 75, and 18.5 ng/µl) induced rapid and prolonged decrease of male responsiveness. The responsiveness significantly decreased 1 h after treatment. Male responsiveness significantly decreased only 2 h after exposure to imidacloprid at the dose of 38.5 ng/µl. No recovery was exhibited after imidacloprid treatment, regardless of the dose: males exhibited responsiveness level lower than that of untreated ones within 72 hours test period. However, maximum effect of different imidacloprid doses (i.e. the lowest responsiveness level) was exhibited at different time intervals: at the dose of 75 and 18.5 ng/µl the maximum effect exhibited 24 h after treatment; at the dose of 38.5 ng/µl after 48 h; and at the highest tested dose (150 ng/µl) 96 h after treatment.

Deltamethrin treatment at the dose of 1.5 ng/µl induced a significant difference in only one of five tested MCrS parameters. Mean values of dominant frequency significantly differed between pre- and post-treatment trials of 1, 48 and 72 h, however the effects were non-uniform: deltamethrin decreased and increased the mean values. On the other hand, imidacloprid treatment affected the quality of male responses to a greater extent. The effects were dose-, time- and parameter-dependent. Decreasing of the MCrS pulse train duration and the number of MCrS pulse trains were observed after treatment of different doses at different time intervals. Differences in pulse train duration were induced by imidacloprid treatment at all doses. The maximum effect was observed at the highest dose of 150 ng/µl, when males emitted significantly shorter MCrS pulse trains at all observed time intervals (except the 3 h trial). At lower doses (75–18.5 ng/µl) males emit significantly shorter MCrS pulse trains 24 h after exposure to insecticide. On the other hand, imidacloprid treatment resulted in prolonged response latency 1 and 2. Imidacloprid affected the dominant frequency of MCrS pulse trains, again the effects was non-uniform: treatment with 38.5 ng/µl increased the mean value of dominant frequency, but dominant frequency decreased at the dose of 75 and 18.5 ng/µl.

Table 39: The effects of imidacloprid (150, 75, 38.5 and 18.5 ng/µl) and deltamethrin (1,5 ng/µl) treatment on the *Nezara viridula* males A significant decrease in the parameter values is indicated with minus (-), a significant increase with plus (+) symbols, and no significant difference with NS. Effects of imidacloprid (at the dose of 150 ng/µl) on MCrS dominant frequency were not analysed.

	1 h	2 h	3 h	24 h	48 h	72 h
General responsiveness						
150.0 ng IMI/µl	–	–	–	–	–	–
75.0 ng IMI/µl	–	–	–	–	–	–
38.5 ng IMI/µl	NS	–	–	–	–	–
18.5 ng IMI/µl	–	–	–	–	–	–
1.5 ng DELT/µl	NS	–	–	–	NS	NS
Duration of the MCrS pulse trains						
150.0 ng IMI/µl	–	–	NS	–	–	–
75.0 ng IMI/µl	NS	NS	NS	–	NS	NS
38.5 ng IMI/µl	+	NS	NS	–		NS
18.5 ng IMI/µl	NS	NS	NS	–	NS	–
1.5 ng DELT/µl	NS	NS	NS	NS	NS	NS
Number of MCrS pulse trains						
150.0 ng IMI/µl	NS	NS	NS	NS	–	NS
75.0 ng IMI/µl	–	NS	NS	NS	NS	NS
38.5 ng IMI/µl	–	–	NS	–		NS
18.5 ng IMI/µl	NS	NS	NS	–	NS	NS
1.5 ng DELT/µl	NS	NS	NS	NS	NS	NS
Response latency 1						
150.0 ng IMI/µl	NS	NS	NS	NS	+	NS
75.0 ng IMI/µl	NS	NS	NS	NS	NS	+
38.5 ng IMI/µl	NS	+	NS			NS
18.5 ng IMI/µl	+	NS	NS	NS	NS	NS
1.5 ng DELT/µl	NS	NS	NS	NS	NS	NS
Response latency 2						
150.0 ng IMI/µl	NS	NS	NS	NS	+	NS
75.0 ng IMI/µl	NS	NS	NS	NS	NS	NS
38.5 ng IMI/µl	NS	NS	NS			+
18.5 ng IMI/µl	NS	NS	NS	NS	+	NS
1.5 ng DELT/µl	NS	NS	NS	NS	NS	NS
Dominant frequency of pulse trains						
75.0 ng IMI/µl	NS	NS	NS	–	NS	NS
38.5 ng IMI/µl	NS	NS	+			NS
18.5 ng IMI/µl	NS	NS	NS	–	–	NS
1.5 ng DELT/µl	–	NS	NS	NS	+	+

IMI=imidacloprid, DELT=deltamethrin

4.5 Experiment 3-Effects of insecticides on male preference for two parameters of the FCS

4.5.1 Effects of imidacloprid on male preference for duration of the FCS pulse train

As in experiment 1, no significant difference in the responsiveness of untreated males was observed when stimulated with the synthesised FCS of pulse train duration shorter than of that characteristic of the natural FCS (i.e. 500, 600 ms) and when stimulated with the natural FCS (Fisher's exact test). Males responded in 76.2% (16/21) to 600 ms pulse train duration and 95.2% (20/21) of them responded to the natural FCS stimulation (Fisher's exact test, P=0.186, NS). Males stimulated by pulse trains of 500 ms duration exhibited 60% (12/20) responsiveness, and 90% (18/20) responsiveness was recorded during natural FCS stimulation (Fisher's exact test, P=0.065, NS). Figure 29a shows the imidacloprid (38.5 ng/µl) effect on male preference for the pulse trains of 600 ms duration. The percentage of males responding to either 600 ms pulse trains or the natural FCS sequence did not significantly differ during pre-treatment and 1 h post-treatment trial (600 ms: 76.2% pre-treatment trial vs. 57.1% (12/21) post-treatment trial, Fisher's exact test: P=0.342, NS; natural sequence: 95.2% vs. 71.4% (15/21), Fisher's exact test: P=0.093). Figure 29b shows the imidacloprid effect on male preference for the 500 ms pulse train duration. When stimulated with the natural FCS no significant difference was recorded in male responsiveness before and 1 h after treatment (90% vs. 75% (15/20), respectively, Fisher's exact test, P=0.408). However, after exposure to imidacloprid, significantly lower percentage of males responded to the stimulation of 500 ms pulse train duration (60% (12/20) response in pre-treatment trial vs. 20% (4/20) response in post-treatment trial, Fisher's exact test, $P<0.05$). Results show that imidacloprid treatment significantly reduced the tolerance for variability of FCS pulse train duration and narrowed the effective range of duration values.

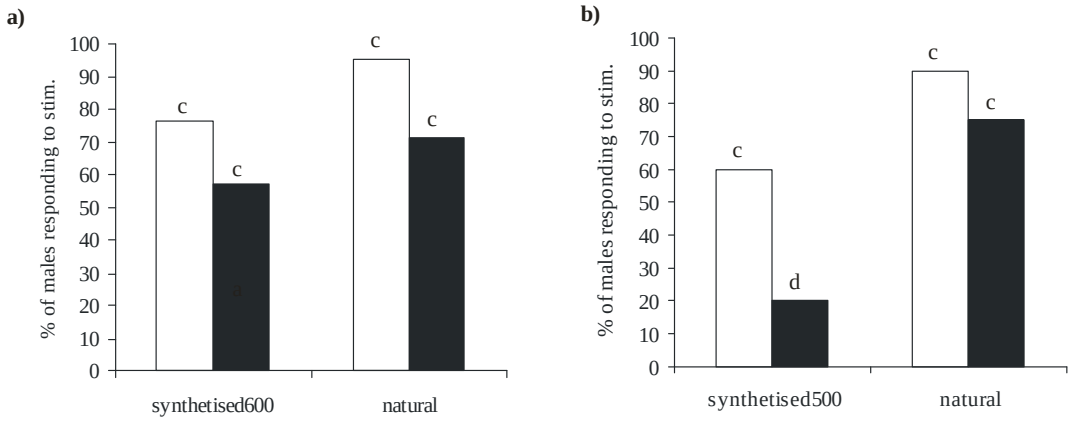

Figure 29: Effects of imidacloprid (38.5 ng/µl) on *Nezara viridula* male preference for duration of the FCS pulse trains 1 h after exposure to insecticide. Percentage of males responding to the natural and synthesised FCS pulse trains of variable duration (a) 600 ms (b) 500 ms. The percentage of responding males to the stimulation of changed pulse train duration and natural FCS sequence were compared between pre-treatment (white bars) and post-treatment trials (black bars) (Fisher's exact test). Different letters indicating significant difference between pre- and post-treatment trials.

4.5.2 Effects of imidacloprid on male preference for dominant frequency of the FCS pulse train

The responsiveness of males was high in all pre-treatment trials: no significant differences in male responsiveness to stimulation with pulse trains of variable dominant frequencies and the natural FCS pulse trains were recorded (90 Hz (100%, 20/20) vs. natural (100%, 20/20) Fisher's exact test: P=1.000; 150 Hz (88.8%) vs. natural (94.4%) Fisher's exact test: P=1.000) (Figure 14). After imidacloprid treatment (38.5 ng/µl) responsiveness significantly decreased only, when males were stimulated by pulse trains of dominant frequency of 90 and 150 Hz. In the pre-treatment trial all males responded to 90 Hz pulse trains, but 1 h after exposure to insecticide responsiveness significantly decreased to 60% (12/20) (Fisher's exact test, P<0.05, Figure 30a). In the pre-treatment, males stimulated with 150 Hz pulse trains showed 88.9% (16/18) responsiveness, however in post-treatment trial the responsiveness significantly decreased to 16.7% (3/18) (Fisher's exact test, P<0.001, Figure 30b). Imidacloprid treatment narrowed the effective range of dominant frequency values. In addition, we evaluated the effect of imidacloprid on male tolerance for frequency values below (90 Hz) and above (150 Hz) of that

characteristic of the natural FCS. Sixty percent of males responded to stimulation of 90 Hz pulse trains, but significantly lower responsiveness (16.7%) to stimulation of 150 Hz pulse trains was recorded (Fisher's exact test, P<0.01). Imidacloprid influenced the tolerance for dominant frequency values above that characteristic of the natural FCS to greater extent than for the frequency below that characteristic of the natural FCS.

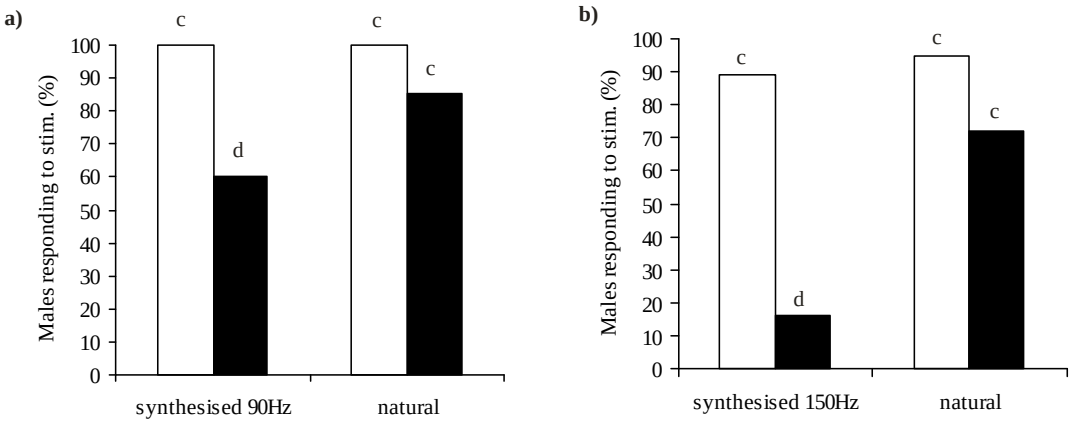

Figure 30: Effects of imidacloprid (38.5 ng/µl) on *Nezara viridula* male preference for dominant frequency of the FCS pulse train 1 h after exposure to insecticide. Percentage of males responding to the natural and synthesised FCS pulse trains of variable dominant frequency (a) 90 and (b) 150 Hz. The percentage of responding males to the stimulation of changed frequency values and natural FCS sequence were compared between pre-treatment (white bars) and post-treatment trials (black bars) Fisher's exact test. Different letters indicating significant difference between trials.

4.5.3 Effects of deltamethrin on male preference for duration of the FCS pulse train

Figure 31 shows male preference for the pulse train duration before and after deltamethrin treatment at the dose of 1.5 ng/µl. Deltamethrin treatment decreased male responsiveness independently of the stimuli characteristics (500, 600 ms duration and natural FCS). Male responsiveness to pulse trains of 600 ms was significantly lower (55%, 11/20) during post-treatment than pre-treatment trial (100%, 20/20) (Fisher's exact test, P<0.01, Figure 31a). When stimulated by the natural FCS, significantly lower responsiveness was recorded during post-treatment (65%, 13/20) than the pre-treatment (100%, 20/20) (Fisher's exact test, P<0.01, Figure 31a). The second group of males, tested for preference for 500 ms duration

(Figure 31b) showed no significant difference in responsiveness between pre- and post-treatment trials. During pre-treatment 71.4% (15/21) of males responded to 500 ms stimulus duration and 57.1% (12/21) during post-treatment trial (Fisher's exact test, P=0.520). Before treatment, 85.7% (18/21) of males responded to the natural FCS and 65% (13/20) responding males was recorded after treatment (Fisher's exact test P=0.158). Results show that deltamethrin treatment did not affect male preference and effective range of pulse train duration, but significantly decreased general responsiveness of males (Figure 31a).

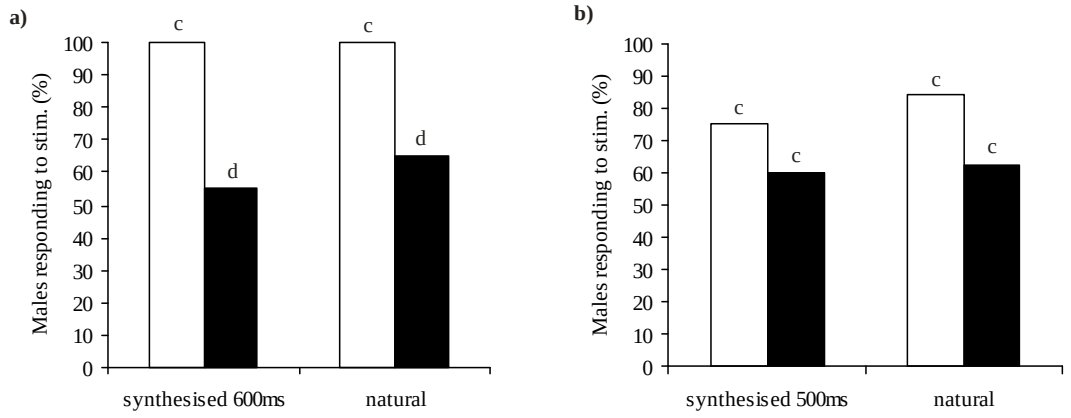

Figure 31: Effects of deltamethrin (1.5 ng/µl) on *Nezara viridula* male preference for duration of the FCS pulse train 1 h after exposure to insecticide. Percentage of males responding to the natural and synthesised FCS pulse trains of variable duration (a) 600 and (b) 500 ms. The percentage of males responding to specific stimulation was compared between pre-treatment (white bars) and post-treatment trials (black bars). Different letters indicating significant difference between trials.

4.5.4 Effects of deltamethrin on male preference for dominant frequency of FCS pulse train

Figure 32 shows male preference for dominant frequency of the FCS pulse train before and after deltamethrin treatment at the dose of 1.5 ng/µl. No significant differences in responses to stimulation with stimuli of variable dominant frequency (90 Hz, 150 Hz) and to the natural FCS before and after deltamethrin treatment were found. Seventy-five percent (15/20) of males responded to 90 Hz stimulus during the pre-treatment and 65% (13/20) during post-treatment trials (Fisher's exact test, NS, P=0.724, Figure 32a). During the natural FCS stimulation 83.3% (15/18) of males

responded before and 88.9% (16/18) after treatment (Fisher's exact test, NS, P=1.00, Figure 32a). During the pre-treatment, 95% (19/20) of males responded to 150 Hz stimulus and during the post-treatment 75% (15/20) responsiveness was recorded (Fisher's exact test, NS, P=0.182, Figure 32b). When males were stimulated with the natural FCS 100% (20/20) responsiveness was recorded during the pre-treatment and 75% (15/20) responsiveness during post-treatment trial (Fisher's exact test, NS, P=0.106; Figure 32b). Deltamethrin did not induce a change in preference and effective range of dominate frequency values of the FCS pulse trains.

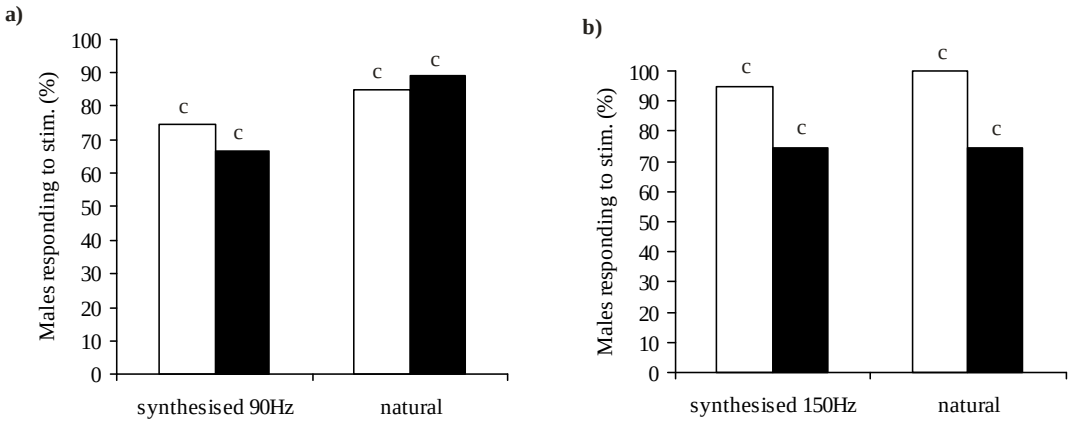

Figure 32: Effects of deltamethrin (1.5 ng/µl) on *Nezara viridula* male preference for dominant frequency of the FCS pulse train 1 h after exposure to insecticide. Percentage of males responding to the natural and synthesised FCS pulse trains of variable dominant frequency (a) 90 and (b) 150 Hz. The percentage of males responding to specific stimulation was compared between pre-treatment (white bars) and post-treatment trials (black bars). Different letters indicating significant difference between trials.

4.6 Experiment 4-Effects of sublethal doses of imidacloprid and deltamethrin on fecundity and the copulatory success of *N. viridula* males

Copulatory success of control and insecticides treated males is shown in Table 40. After imidacloprid treatment (38.5 ng/µl) 60% (12/20) of males responded to FCS stimulations, five out of 12 responsive males, copulated and one copulating male did not respond to FCS stimulation. In control males, and males treated with deltamethrin, males exhibited 100% responsiveness to the FCS stimulation. In the control treatment all (12/12) males copulated, but significantly lower percentage

(30%, 6/20) of imidacloprid treated males copulated (Fisher's exact test, P<0.001). Males treated with deltamethrin (1.5 ng/µl) copulated in 66.7% (8/20); the difference was not statistically significant from that of the control (100%) (Fisher's exact test, P=0.093). Seven days, multiple deltamethrin treatment greatly affected survival of the treated bugs: 65% (13/20) mortality was recorded after 7 days of deltamethrin exposure. Males that did survive exhibited 100% responsiveness to the FCS stimulation and copulations were recorded with all seven males. The number of mating/male was significantly different from that of the control only in the case of imidacloprid treatment: the mean number of copulations in imidacloprid treated males was 1, while with control males 2.4 copulations/male was recorded (Steel test, P<0.05, Table 40). No significant differences were found in copulation duration regardless of the treatment: mean copula duration ranged from 1.4 to 2 days (Steel test, P<0.05, Table 40).

Table 40: Copulatory success of control males and males topically treated with insecticides: imidacloprid (38.5 ng/µl) and deltamethrin (1.5 ng/µl, 7 day multiple deltamethrin treatments).

Treatment	Copulating males (%)	No. of males responding to FCS stim.	No. of mating/male (mean±SD)	Copula duration (day) (mean±SD)
Imidacloprid (38.5 ng/µl)	**30.0*** **(6/20)**	5/1	**1.0 ± 0.0***	1.4 ± 0.5
Deltamethrin (1.5 ng/µl)	66.7 (8/12)	12/0	1.6 ± 0.5	2.0 ± 1.1
Deltamethrin (7 x 1.5 ng/µl/day)	100.0 (7/7)	(7/0)	1.3 ± 0.5	1.8 ± 0.9
Control (1µl distilled water)	100.0 (12/12)	12/0	2.4 ± 1.3	1.7 ± 0.8

Data on fecundity of females that mated with treated or control males are shown in Table 41. Females mated with the control males exhibited the highest oviposition (91.7%, 11/12); oviposition rate in females mated with insecticide treated males (83.3–57.1%) did not significantly differ from that of the control (imidacloprid treatment vs. control: Steel test, P=1.000; deltamethrin vs. control: Steel test, P=0.537; multiple deltamethrin vs. control: Steel test, P=0.117). The mean number of egg masses/ovipositing female in the insecticides treated groups did not differ

significantly from the mean number of the control group (imidacloprid vs. control: Steel test, P=0.057; deltamethrin vs. control: Steel test, P=0.051; multiple deltamethrin treatments vs. control: Steel test, P=0.199).

Comparison of number of eggs/egg mass between the control and insecticide treatments, showed no significant differences (imidacloprid vs. control: Steel test, P=0.133; deltamethrin vs. control: Steel test, P=0.099; multiple deltamethrin treatment vs. control: Steel test, P=0.492). However, the mean number of first filial generation adults (F1) from insecticides treated males was significantly different from that of the control (i.e. 37 ± 20.5). No F1 adults developed from eggs oviposited by females mated with imidacloprid treated males (Steel test, P<0.05), nymphs did hatched from the eggs but all died before reaching the adult stage. On average two (± 3.9) F1 adults developed from males that was single treated with deltamethrin (Steel test, P<0.01), and the mean number of F1 adults from males exposed to multiple deltamethrin treatment was 2.5 (Steel test, P<0.05).

Table 41: Fecundity rate in the control *Nezara viridula* pairs and in pairs of males exposed to insecticide and paired with untreated females. Males were treated with either imidacloprid (38.5 ng/µl) or deltamethrin (1,5 ng/µl single or multiple treatments over 7 days period).

Treatment	Oviposition (%)	No. egg mass/female (mean ± SD)	No. eggs/egg mass (mean ± SD)	No. ad F1/ovipositing female (mean ± SD)
Imidacloprid (38.5 ng/µl)	83.3 (5/6)	1.2 ± 0.5	56.6 ± 6.1	**0.0***
Deltamethrin (1,5 ng/µl)	75.0 (6/8)	1.5 ± 0.5	82.3 ± 12.2	**2.0 ± 3.9****
Deltamethrin (7x1,5ng/µl)	57.1 (4/7)	1.7 ± 0.5	64.1 ± 17.3	**2.5 ± 2.5***
Control (1µl distilled water)	91.7 (11/12)	3.6 ± 2.1	71.4 ± 25.3	37.0 ± 20.5

F1 = first filial generation

5 DISCUSSION

Insecticides at levels that do not lead to mortality can influence behaviour (Haynes, 1988). Pest management practise depends on insecticides and is likely to remain so as long as effective and inexpensive chemicals are available. However, many insect-pest management techniques, based on non-lethal mechanisms, have been successful in population control. The objective of integrated pest management is not directed specifically to kill pests, but to limit their populations below the threshold determined by economic factors and environmental concerns. Insecticide effects on life histories and population fitness, modification of mating, host-finding, feeding and other behaviours are employed to derive better estimates of insecticide impacts on both target and non-target species and may help to provide new avenues toward effective pest management (Haynes, 1988, Stark and Banks, 2003).

Reproduction involves a complex series of behavioural and physiological events, which are coordinated by the insect nervous and hormonal systems. Insecticides can disturb this coordination and diminish the reproductive success. Most often is documented only the end results of sublethal poisoning, which is a decrease in production of viable offspring. Insecticides can decrease production of offspring in different ways, such as reduction in oviposition, associated physiological process (spermatogenesis, oogenesis, ovulation, egg fertilization, etc), affecting mating behaviours (communication between mates, mate-recognition and mate-location process, courtship behaviour, etc) (Haynes, 1988).

There are good examples of how insecticides interfere with mating behaviour mainly focusing on chemical communication. Investigation of the sublethal effects on chemical communication and other behaviours that influence reproduction success is important for understanding and increasing the effectiveness of pest management strategies. For example lepidopteran attracticide formulation (combining pheromone and insecticide component), was shown to be effective not only because of direct lethal effects of the insecticide, but even more on account of its sublethal modification of mating behaviours (Krupke et al., 2002, Evenden et al., 2005). On

the other hand, understanding of sublethal effects on behaviour may help in diminishing insecticide effects on non-target organisms, parasitoids and predators.

Along with chemical signals, air-borne and substrate-borne (i.e. vibrational) signals are very important for the reproduction because they are involved in communication during mating behaviour of many insect species (review in Drosopolous and Claridge, 2006). Species specificity, location and communication range are encoded in temporal and frequency characteristics of the vibrational signals. Compared to air-borne sound communication, vibrational signals are more prevalent among insects (Cocroft and Rodriguez, 2005) but less investigated. Conspecific song recognition has been extensively investigated for communication with airborne signals in Orthoptera. These studies indicate high informational value of different temporal parameters of the signals: absolute pulse duration and/or interval between pulses (Hennig, 2003, Schul and Bush 2002), pulse rate (Hennig and Weber, 1997, Schul and Bush, 2002), duty cycle (Schul, 1998), or/and some combination of these parameters (Doherty, 1985, Doolan and Young, 1989). Doolan and Young (1989) demonstrated that in cicadas carrier frequency of the signal is important for long-range species recognition, whereas temporal parameters become important during short range courtship. Informational values of temporal and spectral characteristics of insect vibrational signals were mainly investigated in Auchenoryncha. The pulse repetition time has been found to be the most important species-specific parameter in leafhoppers and planthoppers (Claridge, 1990, Den Bienman, 1986, DeVrijer, 1986). Studies of vibrational communication in treehoppers on the other hand, showed that signals of different species were distinguished by several signal traits: carrier frequency, duration and pulse repetition time (Rodriguez et al., 2004, Rodriguez et al., 2006, Rodriguez and Cocroft, 2006). In contrast, little is known about specificity of vibrational signals and conspecific song recognition in Heteroptera in which the process of mating recognition system is mediated not only by vibrational signals (as in Auchenoryncha), but also by chemical signals (pheromones) (Millar, 2005, Guarino et al., 2008).

In *N. viridula* male pheromone acts as long range attractant for females (Aldrich et al., 1987, Borges et al., 1987, Brezot et al., 1994). Female on the other hand, initiates

vibrational communication and it is the male, which has to recognize the conspecific female calling song (FCS) and respond to it by emitting male vibrational signals. Although females produce recognition signals in many species female signalling behaviour has been neglected in theoretical studies (De Winter, 1992).

5.1 Preference of *N. viridula* males for FCS parameters and mechanism for recognition of the conspecific song

Male *N. viridula* responded with a short latency and well defined vibrational signals to the natural and artificially synthesised FCS. As such, male vibrational response offers a suitable model system to study male preference for FCS parameters.

Few studies on the effect of the female signal variation on male-mate choice, were carried out and proved that differences between species and population influence male-mate choice in planthoppers (De Winter, 1992, De Winter and Rollenhagen, 1990, Claridge and De Vrijer, 1994) and green lacewings (Henry et al., 2002).

Earlier observation in the southern green stink bug have indicated that FCS is part of the recognition system and that males are able to distinguish between the conspecific and the heterospecific FCS signals, differing in temporal parameters (duration and repetition time) (Čokl et al., 1978, Miklas et al., 2001, Hrabar et al., 2004, Miklas et al., 2003a). In the present study we evaluated male preference for the specific female song parameter, independently (pulse train duration, interval, repetition time, duty cycle and dominant frequency) and investigated which of the FCS parameters are used by *N. viridula* males to recognize the conspecific song. The experiments were conducted on the loudspeaker membrane to avoid signal occurring during transmission through plants (Michelsen et al., 1982).

Males responded to FCS stimulation with the calling (MCS) or the courtship (MCrS) song. Emission of the MCS was less selective than of that MCrS: more males emitted only MCS when stimulated with signals of parameter values outside the range characteristic of the natural FCS. This phenomenon was observed also in the study of

Čokl and co-workers (1978). Males were exposed to stimulation with the female song of the sympatric species *Palomena prasina* L. and *P. viridissima* Poda, which triggered only MCS responses. In the present study we observed that young males, 2-5 days after the final moult, and males just taken from the diapause conditions, emitted MCS more often than MCrS signals. But later with age, males produced more MCrS, and MCS was often just a transitional form to the courtship signal. Shorter signals (as MCS in our case) are less energy consuming and provide less predation risk but on the other hand provide less information to the females (Bailey, 2003). Results from this and previous studies indicate that MCS signal has low species specificity and its role in the mate communication is not clear yet. Males also emit MCS at close range, during antennation and head-butting, which might indicate motivation level of the male and could also be important for the close range female-mate choice. In addition, more than half of tested males emitted less specific MCS also when stimulated with the natural female courtship song (FCrS). Less specific response of males and lower preference for the natural FCrS could be explained by its temporal characteristics. They show higher variability, than that of the FCS, resemble the temporal structure of the MCrS (Čokl et al., 2000), which could inhibit emission of the MCrS and/or provoke MCS emission. Furthermore, in the Slovene population of *N. viridula*, FCrS is present in the vibrational repertoire of females but its emission is restricted to the beginning of the female vibrational signalling and is eventually transformed into FCS (Pavlovčič, 2005). On the other hand, in the Californian population FCS and FCrS are present in the same proportion, in the Australian and probably Guadeloupe population only FCrS is present in the vibrational repertoire of females (Ryan et al., 1996, Jeraj and Walter, 1998, Mikals et al., 2003a). In order to elucidate the role of FCrS in female vibrational repertoire, additional behavioural experiments with females and playback experiments with males should be performed.

Responses of *N. viridula* males were evaluated in relation to the parameter values of the stimuli. General responsiveness (i.e. percentage of responding males to specific stimulus) was found to be the most selective and reliable indicator of male preference for FCS parameter values. On the other hand, signalling activity of responding males did not show high tuning with the female song characteristics. Males exhibited the

maximal difference in signalling activity when they were stimulated with stimuli of variable pulse train duration and constant values of interval and dominant frequency (kept at the values characteristic of the natural FCS). Variation of the interval and dominant frequency did not influence the signalling activity of responding males. Signalling activity of males exposed to stimulation with signals of different interval and frequency values changed more on the account of inter-male variation than on variation of interval and frequency values.

Data on general responsiveness showed that males were clearly able to distinguish FCS of different temporal and spectral parameters. The preference function in *N. viridula* males depended upon which aspect of the FCS was varied. Optimal responses (the highest percentage of responding males) were elicited by stimulation with the artificially synthesised songs of parameter values, resembling those of the natural conspecific FCS. Asymmetry of the preference response was observed for pulse train duration and repetition time (Figure 33). Compared to parameter values longer than values characteristic of the natural FCS, males were more discriminating to values shorter than of those characteristic of the natural FCS. This is in agreement with the study of Čokl et al. (1978) in which they suggested that a sharper decrease of response to shorter parameter values is due to their resemblance to the conspecific male calling or rival song, which both inhibit rival male singing. Furthermore, with decreasing of the parameter values, the stimulation signals resemble more to parameters characteristic of either male or female calling song described for some other pentatomid species (*Palomena prassina, P. viridissima, Acrosternum hilare* Say, *N. antennata* Scott (Čokl et al., 1978, Čokl et al., 2001, Kon et al., 1998), *Tyantha pallidovirens* Stål, *T. custator accera* McAtee ((Hrabar et al., 2004), *Chlorochroa uhleri* Stål (Bagwell et al., 2008). Thus, fewer males responded to stimuli of characteristics resembling the heterospecific songs. Moreover, shorter signal carries less information about the signal, its specificity and directional information (Bailey, 2003). Longer signals may evolve when mate choice has a prominent role relative to factors of minimizing predation risk. In addition, echoes due to standing wave conditions on a plant (Michelsen et al., 1982) prolong transmitted signals, thus justify higher tolerance for the longer signals.

The preference toward longer values was not open ended and effective preference range was determined for all parameters tested. Geographically isolated population of *N. viridula* (Slovene, Italy, Florida, Brazil, California, France, Guadeloupe and Japan) differ in the temporal characteristics (Ryan et al., 1996, Čokl et al., 1999, 2000, 2001, Kon et al., 1988, Jeraj and Walter, 1999, Miklas et al., 2003a) and these differences seem to be genetically determined (Virant-Doberlet et al., 2000, Pavlovčič, 2005). Values of the FCS pulse train duration documented for different populations varied from 752 to 1924 ms, interval from 1912 to 7090 ms and repetition time from 2691 to 8730 ms. Iin different studies relatively large variation in temporal characteristics was reported for Slovene population: pulse train duration varied from 999 to 1924 ms, interval from 1912 to 3469 ms and repetition time from 2691 to 5393 ms. The distribution of mean preference for different parameter values evaluated in the present study coincide with distribution of the parameter values characteristic of the natural FCS of Slovene population documented in the literature (Figure 33). These data indicate that males use these parameters in the process of species recognition and that evolution of the female song is subjected to male selective pressure.

Bimodal preference was observed for pulse train duration with optimal response within the effective duration range between 600 and 1000 ms and the second peak in responsiveness shown at the natural FCrS stimulation, but interestingly at the synthesised stimulus of values characteristic of the natural FCrS, responsiveness was significantly lower from that of the natural FCS. Bimodal preference response was observed for the inter-pulse train interval and repetition time as well. This is not in agreement with previous studies (Čokl et al., 1978, Miklas et al., 2001), in which the authors observed unimodal preference function for the repetition time. The difference is probably due to the experimental setup. They tested different range of parameter values. The maximum repetition time tested was 5000 and 4000 ms, respectively, but the maximum tested repetition time in the present study was 14545 ms. Furthermore, they did not test preference for interval independently, they varied duration within constant repetition time, therefore the interval changed as well. Effective preference range of the interval evaluated in the present study ranges between 1500 and 3000 ms, with the second peak in responsiveness at 7000 ms. The preference range of

repetition time was between 2200 and 3700 ms, second peak was exhibited around 7000 ms.

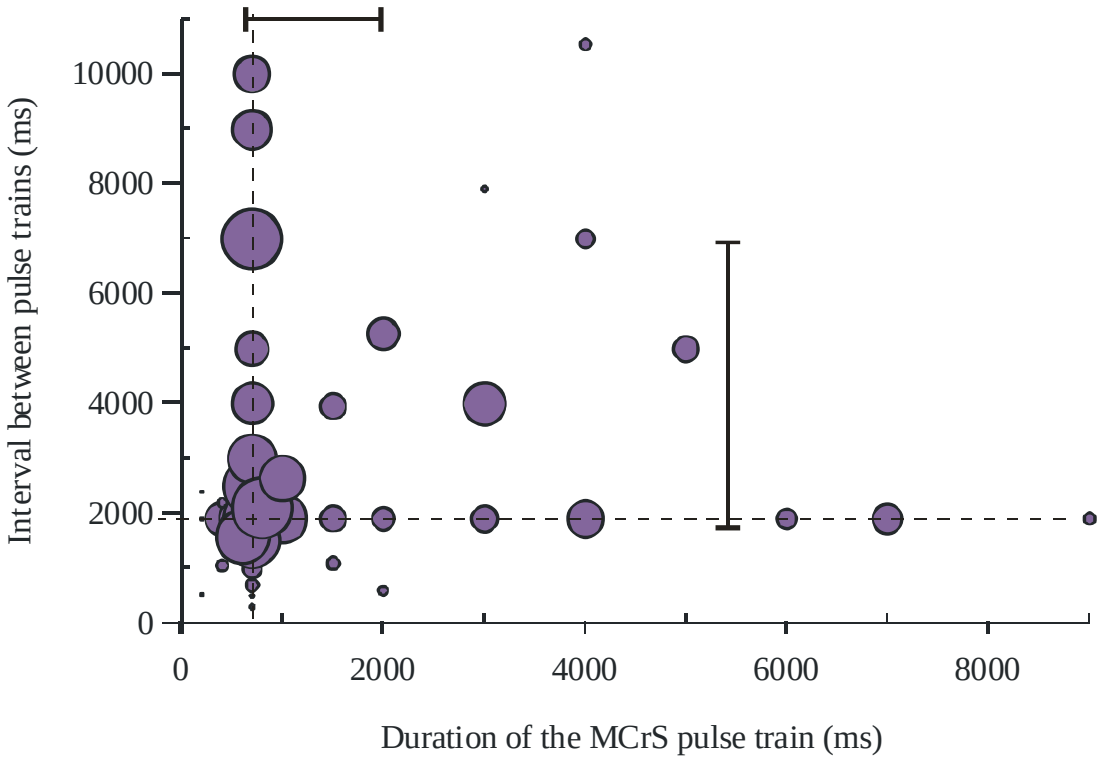

Figure 33: Dependence of vibrational responses of *Nezara viridula* males to the female calling song (FCS) of variable pulse train duration and inter-pulse train interval (interval). The circles give the percentage of responding males to respective parameters combination. Dashed line indicates the reference value used in our experiments (i.e. mean value calculated for the pre-recorded five females from the Slovene population). Black lines indicate distribution range of parameter values described for geographically isolated population (from the literature: Ryan et al., 1996, Čokl et al., 1999, 2000, 2001, Kon et al., 1988, Jeraj and Walter, 1999, Miklas et al., 2003a).

Males responded best to stimulus signals defined by pulse train duration and interval corresponding to values characteristic of those of the conspecific song (Figure 33). Duty cycle of 27% and 2600 ms repetition time the values characteristic of the natural FCS evoked the highest responsiveness. Duty cycle and repetition time, characteristic of the natural FCS, became less attractive when obtained by duration and interval values outside their effective preference range. Repetition time was found to be the parameter least affected during transmission through substrate contrary to signal duration, which can be significantly prolonged (Michelsen et al., 1982, Miklas et al., 2001). Repetition time could be influenced by other factors. Čokl and Bogataj (1982), De Vrijer (1984) showed that with increasing temperature the

repetition time of FCS increased and can therefore be less reliable and consistent recognition parameter. Furthermore, in planthoppers repetition time depends on the motivation, physical condition and age of the signaller (Nuhardiyati and Bailey, 2005). In order to avoid temperature effect on the repetition time our experiments were performed at temperature within a narrow range between 21-23°C. Results of the present study indicate that recognition of the FCS functions on the basis of two temporal filters tuned to the values of pulse train duration and inter-pulse train interval (Figure 33). Filtering of repetition time and duty cycle seems to be subordinate in function to their components, that is to pulse train and interval duration. Results indicate that interval is less limiting factor: the responsiveness is influenced to a greater extent when duration is varied, compared to variations of the interval (Figure 33). Furthermore, males were more tolerant to repetition time and duty cycle, obtained by constant duration (corresponding to the natural FCS) and variable interval, than obtained by constant interval and variable duration. Finally, the distribution of interval in different population is slightly broader, compared to distribution of pulse train duration. This is in agreement with the variation in FCS duration and interval measured between females of the pre-recorded population (N=5, n=35; duration CV_{inter}=0.17; interval CV_{inter}=0.28). However, this is in contrast with previous observations indicating higher variation of pulse train duration than of interval (Pavlovčič, 2005). This difference could be due to the effect of different generations.

All, until now investigated pentatomids vibratory signals, have uniform frequency characteristics with the dominant frequency around 100 Hz and harmonic peaks which do not exceed 1000 Hz (Čokl et al. 2001, McBrien et al. 2002, Čokl and Virant-Doberlet, 2003, Čokl, 2006, Moraes-Blassioli et al. 2005). As expected *N. viridula* males showed strong preference for the stimulus of 105 Hz, which is characteristic of the natural FCS. Effective preference range of dominant frequency was observed to be between 90 and 200 Hz. Because studies on leg vibratory receptor neurons of *N. viridula* (Čokl, 1983, Zorović et al., 2008) demonstrated the presence of neurons tuned to higher frequency values, we tested male responses to high frequencies (above 500 Hz) as well. Since we did not obtain any response to high frequencies we can assume that these neurons are not relevant for the species

recognition system. The dominant frequency around 100 Hz of the FCS, recorded on the non-resonant substrate (Čokl et al., 2000) corresponds to the repetition rate of electromyogram potentials recorded from muscles vibrating the abdomen during singing (Amon, 1990). Resonant spectra of vibrations induced by sound in different green plants are characterised by the dominant frequency between 160 and 220 Hz and subdominant peaks not extending above 600 Hz (Čokl et al., 2005). Signal spectrum of a female singing on the plant, contains additional frequency peaks, below and above the dominant frequency. Characteristics of plant vibrations induced by singing insect are reflected in the FCS spectra and determine transmitted signal frequency characteristics. The preference range of dominant frequency values of *N. viridula* male, determined in the present study, enables recognition of the conspecific signals, of which dominant frequency varies among individuals and between geographically isolated populations (Čokl et al., 2000, Miklas et al., 2003a) and is tuned to the mechanical properties of the substrate (i.e. plant, Čokl et al., 2005, 2007). Until now, species specific spectral characteristics have not been determined in any of the investigated stink bug species, hence indicating that frequency characteristics play a minor role in song recognition and are more important to determine velocity-distance relation. Effective transmission is enabled by tuning of signal frequency with the resonant properties of transmission medium (plants) (Michelsen et al., 1982, Čokl et al., 2005, 2007).

Pre-pulse of the FCS is one of the less influential recognition characteristics of the FCS. Males do require the pre-pulse to respond to the female calling song, but the characteristics of the pre-pulse are not sharply tuned to any specific parameter values (either frequency or intensity). This result is consistent with the one of the study of Čokl and co-workers (2007) concerning the amplitude variation of the FCS signal during transmission through the plant. The amplitude of the signal varies with distance in standing wave conditions: the distance between nodes and antinodes decreases with increasing frequency. The pre-pulse contains frequency components higher than of those characteristic of the longer pulse and has lower intensity. Because of this it disappears within noise at certain distances from the source and as such cannot carry reliable species specific information at longer distances.

5.2 Toxicity of deltamethrin and imidacloprid to *N. viridula* males

Present study is the first one to investigate, whether sublethal doses of insecticide influence the behavioural pattern of vibrational communication. More specifically, we investigated the relationship between the insecticidal types (pyrethroid vs. neonicotinoid), the dose of insecticide bugs were exposed to, and the subsequent ability of these male bugs to recognize and respond to vibrational signal of the females.

Pyrethroids and neonicotinoids can be used, among others, as foliar sprays (Elbert and Nauen, 2004). Most spray applications are especially targeted against pests attacking cereals, rice, potatoes, maize, vegetables, cotton and deciduous trees. Effects of the foliar spraying of imidacloprid and deltamethrin on the non-target species have been already documented (Incerti et al., 2003, Stapel et al., 2000, De Cock et al., 1996, Longely and Jepson, 1997).

Commercial products of deltamethrin and imidacloprid insecticides (Decis 2.5 EC and Confidor 200 SC, respectively) were used in the present study. We are aware of the potential effects of the solvents added in the commercial products (Jemec et al., 2007, Thornham et al., 2008, Tišler et al., 2009). However, the insects in the field are exposed to the commercial products, therefore we used them instead of analytical standards of the insecticides. In order to evaluate the sublethal effects of deltamethrin and imidacloprid, different commercial products of the two active ingredients were used in other studies as well (Walker et al., 07, Rogers et al., 07, Elzen, 2001, Delbeke et al., 1997, Rogers and Potter, 2003, Mahdian et al., 2007, Torres and Ruberson, 2004, Smith and Cave, 2006, Ramirez-Romero et al., 2005, Mohaghegh et al., 2000, Abramson et al., 1999, Boina et al., 2009).

On the plant, insects may be topically exposed to fresh sprays while moving on the plant, feeding and searching for host, prey or mate. In order to mimic field conditions we used method of direct topical application treatment using micro syringe. The

immobilizing procedure used did not influence the physiological and behavioural conditions of males. Standard application across individuals of a defined volume of the test compound is easier achieved by topical application than it is by spraying.

Deltamethrin was more toxic to *N. vidridula* males than imidacloprid, topical LD_{50} was lower for deltamethrin than for imidacloprid. Compared with imidacloprid, deltamethrin induced higher mortality in nymphs and adults of the predatory bug *Podisus bidens* L. as well (Mahdian et al., 2007), whereas other reports show that *P. maculiventris* Say and *P. nigirspinus* Dallas are tolerant to deltamethrin (Mohagheh et al., 2000, Picanco et al., 1996). Toxicity data and data on the sublethal effects of deltamethrin and imidacloprid indicate that both insecticides are species specific (Karnatak et al., 2008, Scott-Dupree et al., 2009). Some of the solitary bees were found tolerant or with very low imidacloprid susceptibility (Abbott et al., 2008). Moreover, for a given species, the toxicity of the insecticides changes with the route of exposure and vary in laboratory and field tests (Kunkel et al., 2001, Studebaker and Kring, 2003, Stark et al., 1995, Bailey et al., 2005). Neonicotinoids are found to be highly toxic by direct contact (Scot-Dupree et al., 2009) but in honeybees imidacloprid was shown to be more toxic via oral route than by contact (Suchail et al., 2000). Generally less toxic by contact, foliar applications of deltamethrin could as well have immediate impact on insects.

Our results show, that sublethal doses of deltamethrin and imidacloprid have an impact on vibrational communication. After exposure to deltamethrin and imidacloprid responsiveness of *N. viridula* males to FCS significantly decreased. Time-course of the action depended on the type of insecticide and the dose of treatment. Furthermore, the nature of the male response and tolerance to different parameter values of the FCS was influenced by the insecticide treatments. This could influence the positive feed-back between male and female vibrational duetting.

5.3 Effects of insecticides on male responsiveness to the FCS

5.3.1 General responsiveness of *N. viridula* males after exposure to the insecticide

Vibrational communication depends entirely on the function of the nervous system. In order to respond to stimulation, males need to receive and properly interpret the information that is encoded in the signal.

Deltamethrin treatment at the dose of 1.5 ng/µl substantially decreased the general responsiveness of males. Principal mode of action of deltamethrin is prolongation of the sodium current during membrane excitation. The effects on Ca^{2+} channels, ATPases, acetylcholine and GABA receptors have also been described (review in Nauman, 1990). Due to those multiple actions on the nervous system, it is difficult to point out by which mechanism and at which, peripheral or central neuronal level, vibrational communication is disrupted by deltamethrin.

Deltamethrin is known as fast acting insecticide causing the knockdown effect (Nauman, 1990). Neurophysiological studies show that knockdown effect is caused by poisoning of the peripheral nerves (Salgado et al., 1983) and the lethal effect is due to an irreversible damage to both the peripheral and central neurons, which occurs when poisoning takes long (Gamon and Casida, 1983). In our study, no knockdown was observed after exposure to deltamethrin at the dose of 1.5 ng/µl and we used it in the subsequent behavioural experiments.

Fast action of deltamethrin (as other pyrethroids) could be caused by its lipophilic characteristics, allowing penetration in insect cuticle and partition into and through lipid cellular membrane (Ramoutar et al., 2009). After crossing the barrier of the cuticle, pyrethroids are conveyed by the hemolymph to the target sites where they are accumulated, metabolised or excreted (Ahmad et al., 2006). High protein and lipid

content of the cuticle and/or great sclerotisation is on the other hand, associated with slower rate of penetration flux of the insecticide and thick cuticle may act as a sink for lipophilic compounds (Greenwood et al., 2007). This hypothesis may explain better insecticide tolerance of heavily sclerotised pentatomid predators compared to their soft-bodied caterpillar prey (Yu, 1988). Quick effect of deltamethrin was observed in our study as well. Maximal effect of deltamethrin and the lowest percentage of responding males was exhibited 2 h after the treatment already. Therefore we assume that the properties of the cuticle may not negatively affect penetration kinetics of deltamethrin in *N. viridula.*

Pyrethroids are prone to biochemical detoxification by enzymes such as cytochrome P450 monooxegenases, carboxyl-esterases and glutathione transferases (Yu, 2008). Metabolic process of pyrethroids is similar in insects, mites and mammals (Ruzo et al., 1988). Deltamethrin is degraded to two major metabolites: PBA (3-phenoxybenzoic acid) and 4'-OH-PB acid sulfate (4'-hydroxy-3-phenoxybenzoic acid sulfate) and only the parent compound is considered toxicologically relevant. Full recovery in general responsiveness of *N. viridula* males was observed 48 h after deltamethrin treatment. We assume that recovery in responsiveness is the result of decreased deltamethrin concentration due to enhanced metabolism and detoxification processes. Similar time-course of deltamethrin action was observed in the study Wiles and Jepson (1994): significant differences in the overall behaviour patterns of *Coccinella septempunctata* between untreated and deltamethrin treated plots were found up to three days after deltamethrin application. Recovery of foraging ability was observed in honeybees treated with deltamethrin (Decourtye et al., 2004b). Furthermore, induction of cytochrome P450 had been reported in honeybees after two days of exposure to fluvalinate pyrethroid, which demonstrates that induction of pesticide-immobilising mechanism can occur rapidly (Kezić et al., 1992). However, detoxification and excretion rates, hence insecticide tolerance and ability to recover from pyrethroid poisoning effects, were shown to be age-, sex- and species-specific. Mohaghegh and co-workers (2000) reported higher deltamethrin tolerance of fourth instar nymphs than of adult females of the *P. maculiventris.* Recovery time in the surviving tsetse flies after application of LD_{15} was shorter in females than in males: females were able to move and fly after 20.6 and 30 h, respectively, but males were

able to move after 25.5 h and did not regained the ability to fly (Quinlan and Gatehouse, 1981). *N. viridula*, both nymphs and adults, were shown to be more susceptible to pyrtehroid insecticide than their predator *P. maculiventris* (Vandekerkhove and De Clercq, 2004).

Electrophysiological studies on giant interneuron synaptic pathway of the cockroach documented that imidacloprid has a biphasic effect; first depolarizes and than blocks synaptic transmission at the postsynaptic membrane of cholinergic synapses (Buckingham et al., 1997). Compared with deltamethrin, imidacloprid action on general responsiveness of *N. viridula* males exhibited different time relation. Imidacloprid treatment induced quick and prolonged decrease in responsiveness with only partial recovery (the responsiveness slightly increased, but was significantly below the pre-treatment value within whole 72 h test period). General responsiveness decreased after exposure to all tested sublethal doses but the time-course of imidacloprid action and the potential for recovery of intoxicated *N. viridula* males did not show linear dose-dependency (Figure 34). The kinetics of imidacloprid action was similar for doses of 150, 75 and 18.5 ng/µl. The first manifestation of the insecticide (i.e. decrease in responsiveness), appeared rapidly (1 h after treatment). The second and the strongest effect was exhibited 24 h after treatment at the doses of 18.5 and 75 ng/µl, with partial recovery at 48 h after treatment. At the highest tested dose (150 ng/µl) the kinetics of behavioural effect was prolonged or/and delayed (the maximum decrease in responsiveness was exhibited 96 h after treatment) and partial recovery was observed only 120 h after treatment. On the other hand, kinetics of imidacloprid action at the intermediate dose of 38.5 ng/µl was different from those exhibited at the higher and lower doses. The first manifestation of the insecticide on responsiveness appeared 1 h after treatment, but the significant decrease was exhibited later than with other three doses (i.e. 2 h after treatment). Furthermore, constant decrease in male responsiveness was observed within 48 h test period and after 72 h partial recovery and a slight increase in responsiveness was observed. Time-course of imidacloprid action and potential for recovery might be explained with imidacloprid metabolism. Although imidacloprid is relatively stable in vitro, it is rapidly metabolised in plants, mammals and insects, yielding a number of related compounds. It was shown that imidacloprid is not the only compound to be responsible for insecticidal activity in aphids and honeybees (Nauen et al., 1998, 1999, 2001, Suchail et al., 2003). Two of the imidacloprid metabolites, a 5-hydroxyimidacloprid and an olefin, with chemical structure similar to imidacloprid, exhibit similar or even higher toxicity. Honeybees were treated orally at 25°C and followed the metabolism of imidacloprid. Imidacloprid rapidly metabolised: four to

six hours after intoxication the concentration of imidacloprid drastically decreased whereas concentrations of the two metabolites increased and after 24 h imidacloprid could not be detected any more (Suchail et al., 2003). Furthermore, they observed coincidence of the appearance of the two metabolites and mortality several hours after intoxication. The time-course of imidacloprid action in *N. viridula* males was also consistent with the time of imidacloprid penetration through the isolated cuticle and speed of action of imidacloprid in intact larvae of *Spodoptera littoralis* (Boisduval) following topical exposure (Greenwood, et al., 2007). It is possible that the first decrease in responsiveness of *N. viridula* males tested was caused by fast penetration and toxicity of the parent compound, whereas, after several hours, low or even further decrease in responsiveness could be induced by the two imidacloprid metabolites. Results from previous studies on honeybees and our study with *N. viridula* males suggest that imidacloprid metabolites might contribute to the extending of insecticidal action of imidacloprid in different insect species. The metabolism of the insecticide is dependent on the species and its physiology (e.g. cuticle thickness, nutritive status, metabolic rate) and also on the test conditions (e.g. temperature, insecticide solutions, exposure route). Therefore for detailed comparison of metabolic pathway of imidacloprid in different species all these considerations should be taken into account. In contrast, deltamethrin was characterised again as fast acting insecticide, with the possibility of full recovery within relatively short time after intoxication. No recovery was observed in foraging ability of honeybees treated with imidaclopird (Decourtye et al., 2004b). On the other hand, Kunkel and co-workers (2001) showed that most of the imidacloprid intoxicated beetles, at least in the laboratory, eventually recovered from the imidacloprid intoxication. Discrepancy in results could be explained by the difference in the time period of toxicity evaluation: poisoning effects were followed up to seven days in beetles, but responsiveness of treated bugs were evaluated up to three or four days in the present study. Furthermore, insecticide treatment differed between the two studies: spray application was used with beetles, whereas *N. viridula* males were treated individually with a specific imidacloprid dose.

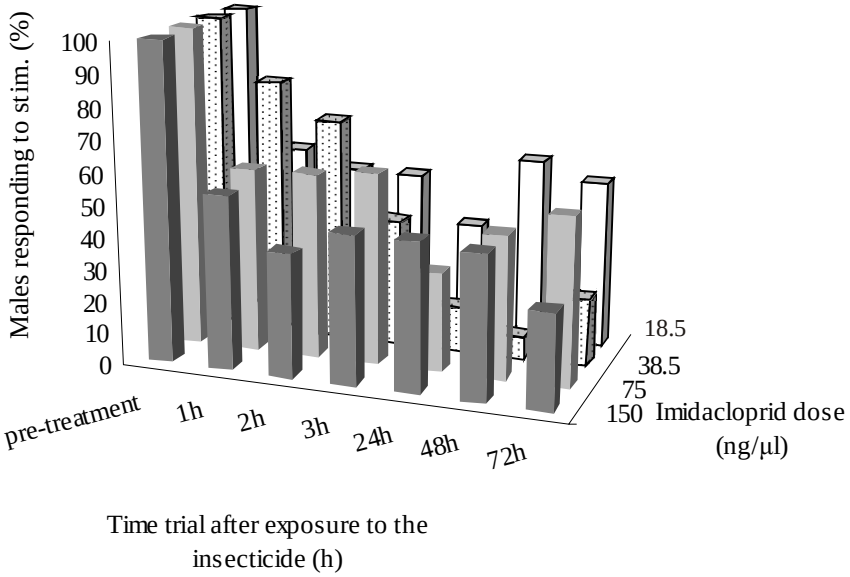

Figure 34: Time-course of imidacloprid action on general responsiveness of *Nezara viridula* males to female calling song stimulation over 72 h time period. Imidacloprid treatment at doses of: 150 (grey bars), 75 (light grey bars), 38.5 (dotted bars) and 18.5 ng/µl (white bars).

Imidacloprid treatment did not induce linear dose-response relation (Figure 34). After exposure to the lowest dose, general responsiveness of males was the least affected (18.5 ng, i.e. 35%), but not significantly less than after exposure to the highest tested dose (150 ng/µl, 21.1%). Surprisingly, maximal effect on the responsiveness (7.1%) and the lowest potential for recovery was observed at the intermediate dose (38.5 ng/µl). These results suggest again that metabolic pathways might be involved in the imidacloprid activity. It is possible that the lowest dose is not high enough to induce detoxification mechanisms, thus the effect on responsiveness is almost the same as the one induced by the highest dose. On the other hand, high imidacloprid action at low dose could indicate the presence of very high-affinity imidacloprid binding sites. In a radioligand binding study by Lind et al. (1998) showed that, in addition to the lower affinity sites that are also seen in insects from different orders, high-affinit [^{3}H] imidacloprid binding sites can be found only in the hemipteran insects and this may explain why imidacloprid is particularly useful in controlling sucking pests. Whereas, the highest dose can activate detoxification enzymes, therefore not increased, but prolonged insecticide action on

the account of high amount of metabolites was exhibited. We assumed that with intermediate dose of 38.5 ng/µl, both parent compound and toxic metabolites induce strong and relatively rapid decrease in responsiveness of *N. viridula* males.

5.3.2 Effects of the insecticides on the quality of male response

Deltamethrin and imidacloprid differently affected responding males and the quality of their responses. Differences observed in the quality of the MCrS response (i.e. in MCrS parameters) may reflect action of the two insecticides at different levels of the nervous system. Deltamethrin at the dose of 1.5 ng/µl influenced only the spectral characteristics of the MCrS. The effects was non-uniform, deltamethrin increased and decreased the dominant frequency of the MCrS. The difference was very small (within 5 Hz) therefore it is probably not relevant for the animal, and would not be perceived as a difference. In addition, the spectral characteristics were analysed for the MCrS emitted during no stimulation period (to avoid overlapping with the stimuli), when males are less motivated and emit fewer MCrS, so the number of the analysed MCrS was not very high. We assume that males, which were able to respond to the FCS stimulation, were not affected by deltamethrin very much. Eventually, they were more tolerant to deltamethrin due to greater vigour or genetically determined susceptibility to insecticide. Fitness may vary as a function of characteristics, such as size, stress, general physical state, but in our study this characteristics were not specifically evaluated. The lack of deltamethrin effect on the quality of the response, on the other hand, suggests that deltamethrin does not disturb the central pattern generator, involved in the production of vibrational signals. Instead, deltamethrin may disturb the process of reception or/and information processing which resulted in a decrease of general responsiveness of males. Some authors reported high deltamethrin sensitivity of sensory neurons (Nauman, 1990, Echobion, 2001). The depolarising action of deltamethrin has a dramatic effect on the sensory nervous system because these neurons tends to discharge even slightly depolarised and can be affected by picomolar concentrations of deltamethrin.

In contrast, imidacloprid influenced the quality of the male responses to a greater extent: the duration and number of the male courtship signals decreased and response latency increased, regardless of the dose. The greatest effect on the temporal patterns of the MCrS was observed at the highest tested dose (150ng/µl) and the duration of the MCrS pulse trains was the most affected parameter. Changes in the temporal pattern of the MCrS response may be attributed to the imidacloprid action on the central pattern generator that coordinates motor neurons innervating the muscles. It has been demonstrated that each cycle of the emitted vibrational signal is preceded by an electromyographic (EMG) signal of timbale muscles and 1:1 relation between EMG signals and spikes in motor neurons which drive timbal muscles was shown (Amon, 1990, 1991). Differences in the quality of the response were generally recorded several hours after the intoxication (with maximal effect at the 24 h time interval) indicating that imidacloprid metabolites rather than the parent compound itself altered the quality of the response. Rapid effect and decreased duration of the MCrS, 1 h after treatment, was observed only at the highest dose. We assume that this effect is caused by direct action of high imidacloprid dose. The effects of short-term and long-term action of imidacloprid and its metabolites, respectively, were documented in honeybees as well (Guez et al., 2001, 2003). Interestingly, imidacloprid treatment at dose of 38.5 ng/µl induced contrasting effects on the quality of the males response, prolonging of its duration. We have no clear explanation for this effect, but a slight activation of the cholinergic system with a low dose of imidacloprid possibly causes a prolongation of the MCrS duration. Similar effects were shown in honeybees and thrips (Lambin et al., 2001, Joost and Riley, 2005). Increased mobility was observed in honeybees treated with lower imidacloprid dose. Imidacloprid altered probing behaviour in two trips species in different ways: in *Frankliniella fusca* Hinds imidacloprid decreased the frequency of probing and ingesting behaviour, whereas *F. occidentalis* Pergande probed and ingested more frequently. The authors proposed different explanations for this effect. First, the contrasting behavioural responses could be attributed to the antagonistic and agonistic effects of imidacloprid. Modulation of the nAChR-chanell by imidacloprid has been demonstrated in rat, planthopper and honeybees, corresponding to both multiple agonistic and antagonistic effects of acetyl-cholin induced currents (Nagata et al., 1998, Deglise et al., 2002, Zhang et al., 2008,).

Second, the contrasting effects of imidacloprid could be associated with the existence of pharmacologically distinct subtypes of nAChR that have different low- and high-affinity nAChR-like binding as demonstrated in in *Myzus periscae* Sulzer (Lind et al., 1998). Imidacloprid induced changes in the dominant frequency of the MCrS pulse trains. As with deltamethrin this differences were very small.

5.4 Effects of insecticides on male preference for duration and dominant frequency of the FCS pulse trains

As expected, due to different chemical structure and mechanism of action, deltamethrin and imidacloprid differently affected the male preference for pulse train duration and dominant frequency. Deltamethrin at the dose of 1.5 ng/µl did not affect male preference for any of the two tested parameters. In contrast, imidacloprid at the dose of 38.5 ng/µl influenced preference for both parameters tested, pulse train duration and dominant frequency.

Males from three out of four tested groups, exhibited no difference in responsiveness to modulated and natural sequence of the FCS stimulation before and one hour after deltamethrin treatment. Only when males were stimulated by FCS of 600 ms pulse train duration, their responsiveness decreased. However, lower responsiveness of treated males was recorded by stimulation with the natural sequence of FCS as well. Therefore, we assume that a decrease in responsiveness to FCS of changed characteristics was not caused by deltamethrin effect on recognition ability of signals of decreased duration, but by its effect on the general responsiveness of males. Deltamethrin affected either receptor cells, or any other part of the sensory-motor neuronal network resulting in decreased general responsiveness.

In contrast to deltamethrin, preferences for both tested parameters were influenced by the imidacloprid one hour after treatment. Prior to imidacloprid treatment high responsiveness of males were exhibited with either decreased stimulus duration (600 and 500 ms) or of that characteristic of the natural FCS (700 ms) stimulus. After imidacloprid intoxication only responsiveness to the shortest duration (i.e. 500 ms)

significantly decreased. We can conclude that treated males were able to recognise modulated FCS pulse trains, but only in a restricted range and the tolerance for duration variability decreased after imidacloprid intoxication. This suggests that imidacloprid may act at the level of information processing and filtering of the temporal parameters, rather than at the level of sensory neurons. Imidacloprid decreased the tolerance for the dominant frequency higher than of that characteristic of the natural FCS, suggesting that imidacloprid can influence peripheral filtering of the frequency characteristics of the signal as well, either affecting the sensory or higher order neurons.

5.5 Effects of insecticides reproductive success of treated *N. viridula* males

Preliminary results of the present study suggest negative effects on reproduction of both deltamethrin and imidacloprid, but further studies are needed to get a clear interpretation of the results.

Male copulating success was impaired by the exposure to imidacloprid and not to deltamethrin, regardless of the time of exposure. One hour after single deltamethrin treatment at the dose of 1.5 ng/µl males exhibited high vibrational responsiveness and high copulating activity. Moreover, no effect on vibrational responsiveness and copulating activity was exhibited also after a long-term (7 days, multiple) exposure to deltamethrin. However, we should take notice that long-term exposure to deltamethrin had lethal character and only seven out of 20 males survived multiple treatments. The long-term exposure can result in the selection of less sensitive males. Such resistant males may give results that are not representative of that of the whole population.

In contrast, single imidacloprid treatment at the dose of 38.5 ng/µl reduced reproductive activity of males; the number of copulating males decreased and mating frequency of specific male reduced. Only half of the males that responded to FCS stimulation copulated, and one unresponsive male copulated. We assume that a decrease in copulation success was not caused only by imidacloprid effect on

vibrational communication, but it could also affected other close-range mating behaviours, circadian rhythm of reproductive activity and/or affect reproductive or other organ systems, determining physical condition and general attractiveness of the males. Effects of multiple imidacloprid treatment remain to be investigated in the future and would allow comparison of long term effects of different types of insecticide.

Fecundity of females (number of eggs laid per lifetime) observed in our experiments was lower but within the range of fecundity of *N. viridula* observed in other study (McLain and Marsh, 1990). No differences in oviposition percentage and fecundity between females paired with control or treated males were observed. These results are not surprising. Fecundity depends preferentially on females fitness, more specifically, their size or body weight that correlates with protein–rich diet (Heming, 1999). Larger or heavier females of stinkbugs showed higher number of egg masses and nymphs than the lighter ones (Oliveira et al., 2005).

Total fertility rate of treated males (regardless of insecticide treatment) was lower than that of control males; very low and zero adults of filial generation (F1) developed from eggs oviposited by females that copulated with deltamethrin and imidacloprid treated males, respectively. High proportion of fertile eggs was observed in pairs of either treated or control males. Imidacloprid may impair the development of nymphal stages. But, we did not systematically count the number of fertilised and hatched eggs, therefore these are just preliminary results and we cannot exclude that insecticide could affect egg fertility resulting in low number of F1 adults as well.

There are contrasting data on the effects of either deltamethrin or imidacloprid on reproductive success of various insect species in the literature. Studies on parasitic wasps reported absence of deltamethrin effects on their reproduction (Deseneux et al., 2005, Garcia et al., 2006), on the other hand, deltamethrin was found to reduce the fecundity in German cockroach (Lee et al., 1998). Different authors documented that exposure to or feeding on sublethal concentrations of imidacloprid reduces reproduction in many Hemipteran insect species (Devine et al., 1996, Bao et al.,

2009, Boina et al., 2009, Widiarta et al, 2001). In contrast, other studies on insect and mite species reported reproductive stimulation by imidacloprid treatment (Elzen et al., 2001, James and Price, 2002). Imidacloprid toxicity strongly depends on the development stage that was applied to, mode, time and its rate of exposure (Elbert et al., 1998). This could be the reason for the contradiction of the results and would warrant caution in generalizing the effects of this two insecticides on reproduction.

5.6 Future work

Future work to be performed in the context of this research should include some of the issues concerning mode of insecticide exposure, species-specific toxicity, behavioural and reproduction effects.

Some authors reported that solvents in the commercial products and active ingredient alone induce different toxicity and behavioural responses in tested insects. Further study using technical standards of the insecticide might elucidate whether this is the case for deltamethrin and imidacloprid and also relevant for vibrational communication.

Because toxicity of the insecticides changes with the route of exposure, further studies should focus on the effects of the given insecticides on vibrational communication after oral exposure (feeding trials) and residual contact.

We demonstrated that selected insecticides adversely affect vibrational communication in *N. viridula* males. However, more work is necessary to investigate whether the insecticides influence the vibrational communication in *N. viridula* females and if the same toxic effect is present in other species, preferentially in the predatory ones as well. Furthermore, insecticide potential to affect other behaviours mediated by substrate-borne vibrations should be explored in the future.

Insecticides act at the peripheral and central level of the nervous system. Playback experiments with modulated and natural stimuli suggested some of the possible

mechanism of the two insecticides actions at different levels of nervous system. Clearly, more studies are required to understand the precise mechanism involved in changes induced by insecticides. In this context, additional behavioural and electrophysiological experiments on insecticides effects on the frequency tuning of the receptor cells should be performed. These data will help to understand, whether insecticide alters the quantity or/and the quality of the signal perceived by the animal, or the specific insecticide interferes with the motor organs determining the quality of the response.

This study investigated the impact of deltamethrin and imidacloprid on the reproductive success of *N. viridula.* Changes in reproduction associated with insecticides may be caused by the effect at the physiological and behavioural level. Reproduction of *N. viridula* could be reduced because of the impact on vibrational communication (species recognition and localisation) or any other step of close-range courtship behaviours (Borges et al., 1987). Therefore, experiments investigating other behaviours (e.g. localisation of the female on the plant, antennation, head-butting, male aedeagal insertion) should be included in future research. Furthermore, effects on circadian rhythm of reproductive activity associated with pheromone production and pheromone communication and/or effect on reproductive or other organ systems, which determine physical condition (analysing feeding behaviour) and general attractiveness of the males, should be investigated. Experiments on feeding activity, analyses of body size and weight fluctuation would give information on physical condition of treated insects. Attractiveness of treated males could be evaluated in the mate choice experiments, in which females would choose between treated and untreated males.

No relation between fecundity and insecticide treatment was observed in our study, but only males were exposed to the insecticide. Experiments with treated females that would be paired with untreated males, would allow clearer interpretations of insecticides effects on fecundity.

Our results point at the reduction in male fertility due to insecticide treatment, but did not examine the insecticide effects on reproduction organs. Dissection of treated

males analysing the size of the testis, spermatozoa condition, its fertilizing capacity and sperm release could elucidate mechanism and effects of insecticides on the fertility.

6 CONCLUSIONS

The results of the present study demonstrated that female calling song (FCS) of *Nezara viridula* is important for the species recognition. *N. viridula* males exhibited two temporal filters tuned to the species-specific values of the pulse train duration and the inter-pulse train interval. Filtering of the repetition time and duty cycle seems to be subordinate in function to their components that is to the pulse train and interval duration. Asymmetry of the preference response was observed for the FCS parameters. Compared to parameter values above that characteristic of the natural FCS, males were more discriminating to values below that characteristic of the natural FCS. The distribution of mean preference for different parameters evaluated in the present study coincided with the distribution of parameter values characteristic of the natural FCS. These results indicate that evolution of the female calling song has responded to selection exerted by the male preference and has been subjected to male selective pressure.

Deltamethrin (a pyrethroid) and imidacloprid (a neonicotinoid) insecticides were proved to affect vibrational communication. Sublethal doses of both insecticide treatments decreased the general responsiveness of *N. viridula* males to FCS. The decrease of male response to FCS might influence the positive feed-back between male and female vibrational duetting and this may cause lower species recognition and mate localisation resulting in a decreased mating rate and reproduction success in the natural population.

It was shown that the time-course of the action depends on the type of insecticide. Deltamethrin induced rapid and strong decrease in responsiveness, with full recovery of the intoxicated males. Imidacloprid, on the other hand, induced prolonged toxicity and decreased responsiveness with low ability to recover. Furthermore, the nature of response (duration of the MCrS pulse trains as the most affected parameter, number of the MCrS and the response latency) and tolerance for FCS parameter values were influenced mostly by imidacloprid. These differences could be associated with specific insecticide type, different target sites and mode of actions at the neuronal level.

This study attempted to establish a dose-behaviour relation for imidacloprid. Adverse effects of imidacloprid in *N. viridula* males were time- and dose-dependent, however, the dose-response relation was not straightforward and further behavioural and electrophysiological studies are necessary to confirm or reject the possible reasons.

The present research demonstrated that male reproductive success differs according to the type of the insecticide males were exposed to. Only imidacloprid suppressed the male copulating success, whereas deltamethrin (regardless of the time of exposure) had no effect on the copulating success. A decrease in total fertility rate (number of adults of first filial generation) was induced by both, deltamethrin and imidacloprid, insecticides. However, it remains to establish whether the suppression of reproduction success is induced by insecticides affecting behavioural or/and physiological processes of treated insect.

Present study confirmed that sublethal effects of insecticides impaired vibrational communication and mating success in *N. viridula*. These results also suggest possible implications for IPM. One implication is that reduced insecticide doses could decrease pest populations because sublethal exposure may interfere with male-female communication. In addition, the lowest tested sublethal dose exhibited the same behavioural modifications as recorded at the highest tested sublethal dose (experiments with imidacloprid). In both cases, application of sublethal doses of insecticide would provide pest control associated with lower negative impact for the environment and other non-target organisms. On the other hand, sublethal doses of insecticide may suppress the populations of heteropteran predatory species, because of the effects on vibrational communication as part of their mating behaviour. Thus, insecticide compatibility for IPM programme should not be based only on the mortality rates. Further implications of the results of the present study could be regarding the possible risks to the population biology of non-target insects in an environment contaminated by insecticides. The decrease in male responsiveness is caused by insecticide effects on the nervous system of the insect, therefore it is not specific only for the communication between mates. The action of the insecticide

could result in interfering with other behaviours in different species using vibrational signalling, such as prey detection, host location, defence and alarm behaviours.

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