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ORIGINAL PAPER

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Effects of imidacloprid on adult and larval stages of the flea *Ctenocephalides felis* after in vivo and in vitro application: a light- and electron-microscopy study

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Abstract The effects of imidacloprid (Advantage®) on the larval and adult stages of cat fleas (*Ctenocephalides felis*) were studied in vivo and in vitro by means of light and electron microscopy. It was found that:

1. The compound acted rapidly on both larval and adult fleas, killing both stages within 20 min of contact.
2. When applied as a *spot-on* to the skin of dogs, the compound localized in the water-resistant lipid layer of the skin surface and in the hairs but not in the blood.
3. Thus, the compound was not taken up during sucking of the flea but was absorbed via the thin intersegmental membranes, since larval and adult fleas that had only external contact with imidacloprid-impregnated paper or with shaved hairs from imidacloprid-treated dogs showed reactions similar to those shown by fleas sitting on treated skin.
4. The compound led to a continuous blockage of insect-specific nicotinic-acetylcholine receptors (nAChR), causing tetanic muscle contractions within minutes of exposure. This manifested as intense trembling of the legs and pumping movements of the body. The affected flea stages remained motionless while the nerves and muscles were constantly and irreversibly destroyed due to hyperactivity. The ganglia of the head and thorax and the striated muscles of the flea body and legs were damaged first, whereas the intestinal movements (e.g., visible in larvae) took longer to exhibit damage.

In summary, these studies show that imidacloprid kills larval and adult flea stages rapidly via the same mode of action and thus prevents the development of flea populations in human or animal dwellings.

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Introduction

The flea is the most common blood-sucking parasite in a wide range of different warm-blooded hosts, including humans, dogs, and cats. The cat flea (*Ctenocephalides felis*) represents about 70% of all flea infestations (Peus 1938; Wenk 1953; Rothschild et al. 1986; Dryden and Rust 1994). Once introduced into a human dwelling, fleas irritate the human and animal inhabitants considerably due to their constant blood-sucking activity. The rapid reproduction rate of the flea results in a new generation every 3 weeks under favorable conditions (Bardt and Schein 1996). If left untreated for a short period the flea populations may become so numerous that eradication requires the consultation of pest-control operators. Humans, cats, and dogs suffer from the consequences of the flea bites, during which saliva and anticoagulants are injected. The latter may lead to severe reactions ranging from erythema, edema, and intense and painful itching to severe symptoms of hypersensitivity or allergy dermatitis in humans and animals (Mumcuoglu and Rufli 1983; Reedy and Miller 1989; Halliwell 1995; Mehlhorn et al. 1995). Besides the above-mentioned symptoms, fleas transmit infections of the tapeworm *Dipylidium caninum*, which is often found in dogs, cats, and, potentially, children (Mehlhorn et al. 1993, 1995). Furthermore, fleas may also transmit a variety of rickettsiae (e.g., *Rickettsia typhi*), bacteria (e.g., agents of plague), or viruses (e.g., agents of hepatitis B, poliomyelitis; Lane and Crosskey 1993). Thus, protection of humans and their companion animals against fleas is essential.

A wide variety of insecticides with adulticidal or larvicidal activity have been developed. The recently introduced compound imidacloprid (a chloronicotinyl-nitroguanidine[1]) is such an excepient, unrelated to any of the other presently used products. Imidacloprid is marketed as a topical solution (Advantage) to be applied

[1] Registered as Advantage®, trademark of Bayer AG, Leverkusen, Germany

as a "*spot-on*" onto the skin of cats and dogs for a 4-week period of protection against fleas (Hopkins et al. 1996; Hanssen et al. 1999). The present study reports experiments on the mode of uptake of this compound by adult and larval fleas and describes its effects inside the body of the flea.

Materials and methods

Parasites

Larval and adult cat fleas (*Ctenocephalides felis*) were reared in the laboratory using standardized methods (Hudson and Prince 1958).

Dogs

Three beagle dogs weighing approximately 13 kg and aged 1.5–2 years were treated with a 10% (w/v) topical formulation of imidacloprid (chloronicotinylnitroguanidine, Advantage; Fig. 1) as a single "*spot-on*" on the neck. The dose was based on the animals' body weight (10 mg/kg). At 7 days after application of the product the dogs were shaved with an electric safety razor to obtain a circular hairless skin region measuring approximately 20 cm in diameter on the lateral side of the body. The shaved hairs were collected in petri dishes and stored for the in vitro experiments.

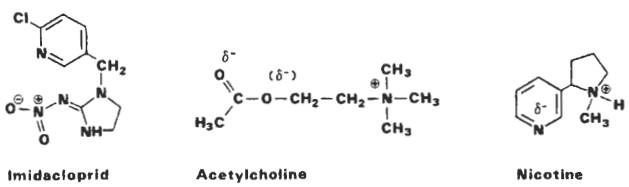


Fig. 1 Structures of three compounds that can block or activate the nicotinic acetylcholine receptor (nAChR) in insects

Figs. 3–6 Light micrographs of semithin sections through the anterior region of the head/body of adult fleas. **Fig. 3** Oblique sections through the nerve system of an in vitro-treated stage, showing lightening of the nuclear region of nerve cells. Beside the central esophagus, tracheoles (*TR*) are present, but there is no alteration in the tissue. ×150. **Fig. 4** Oblique sections through the subesophageal ganglion of an untreated flea; no degeneration is visible. ×150. **Fig. 5** Sections through the thorax of a treated flea. Note the damage in the ventral nerve chord (*NC*) and along the nonsclerotized intersegmental membranes (*IS*). ×100. **Fig. 6** Higher magnification of two intersegmental membranes (*IS*) within a treated adult flea; these membranes interconnect the thorax and the legs. Note that the muscles close to the membranes show degeneration. ×400

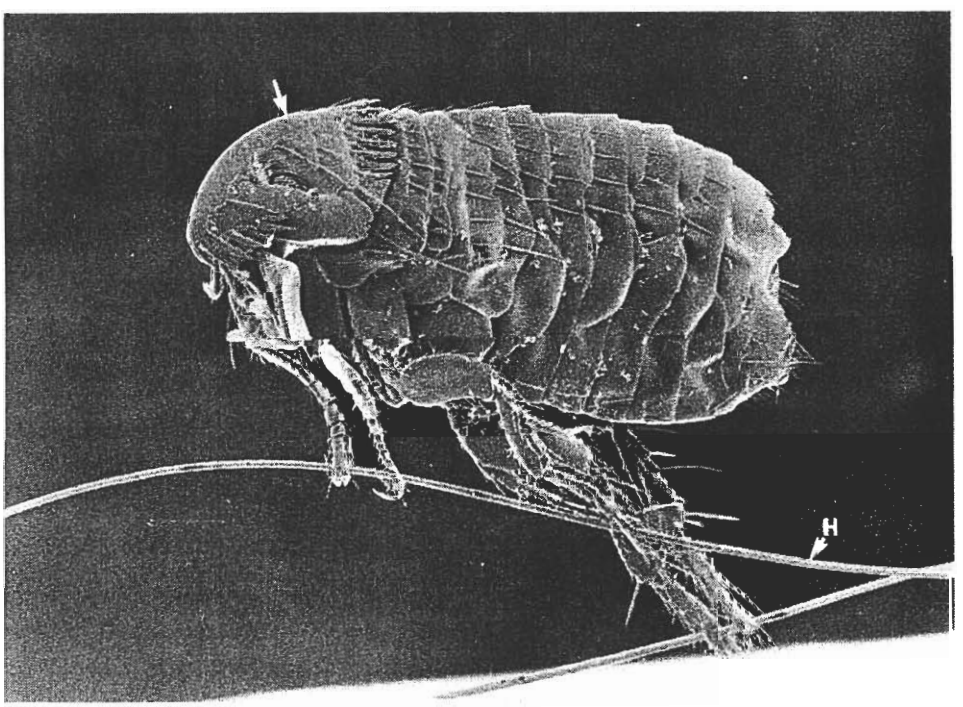
Experiments

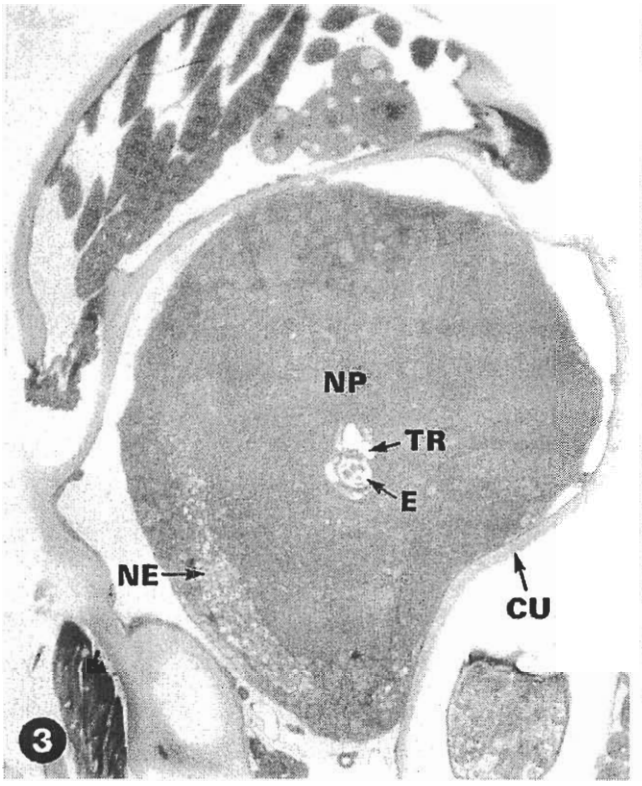
In vivo

A clean cover of a petri dish was fastened with tape to the hairless region. Approximately 100 unfed adult fleas were applied to the shaved area beneath. The behavior of the fleas was observed by a

Fig. 2 SEM micrograph of an adult cat flea, lateral view. The *arrow* shows the main direction of the semi- and ultrathin sections used in this study. ×50

Abbreviations *A* Anterior stomach · *ACh* Acetylcholine · *AX* Axon · *CH* Chromatin · *CO* Collagen fiber · *CV* Cellular cover of the ganglion · *CU* Cuticle · *DA* Degenerating axon · *DC* Degenerating nerve cell · *DM* Degenerating mitochondrion · *DN* Degenerating nucleus · *E* Esophagus with erythrocytes · *F* Feces of adult fleas containing blood · *FI* Fibrillar layer of connective tissue · *H* Hair · *IN* Invaginating tubular systems · *IS* Interseqmental membrane · *MI* Mitochondrion · *MT* Microtubule · *MU* Muscle fiber · *MY* Myosin fiber · *N* Nucleus · *NE* Nerve cell · *NP* Neuropil (layer of axons) · *SG* Salivary glands · *SEM* Scanning electron microscopy · *TEM* Transmission electron micros- copy · *TR* Tracheoles · *VE* Vesicles · *VN* Ventral nerve chord · *Z* Z-line of sarcomere

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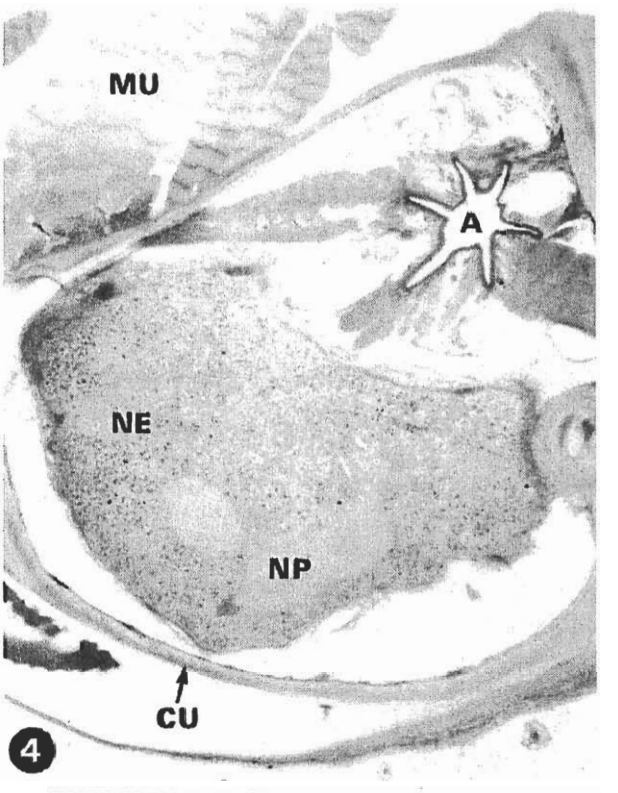
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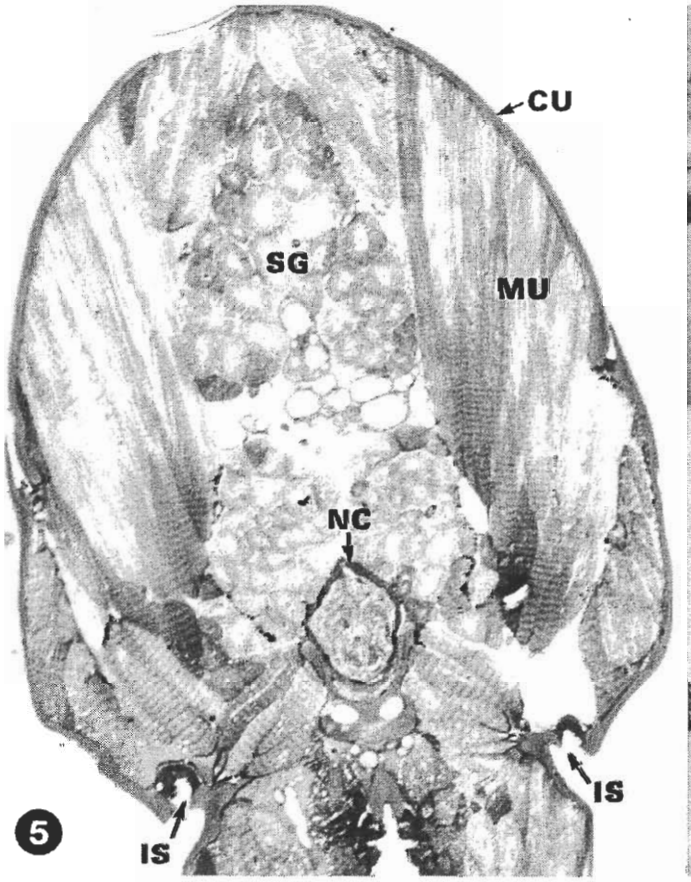
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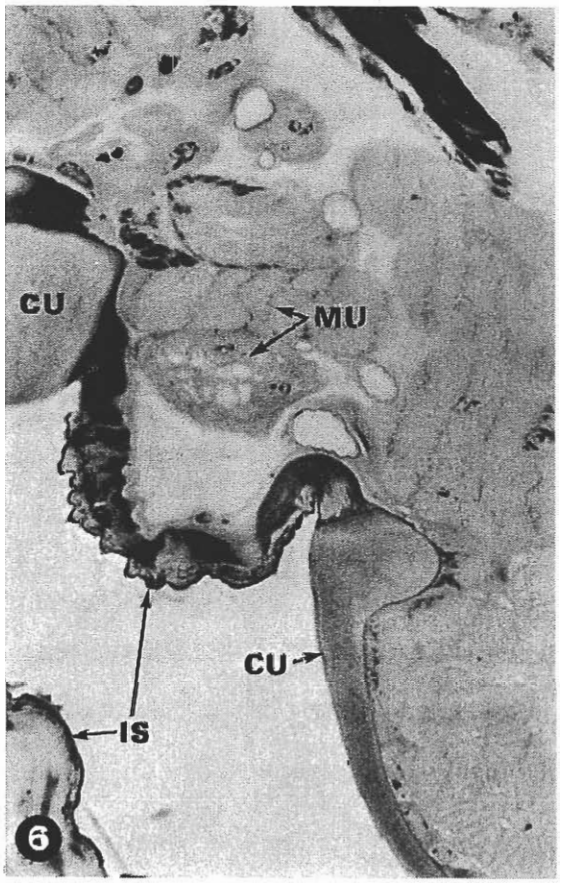
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video camera for 1 h. After this period the adult fleas were collected and fixed for light and electron microscopy. Then the surface fat covering the hairless skin region was removed by repeated intense cleaning with alcohol. A further 100 unfed adult cat fleas were exposed to this area and were observed with a video camera for another hour, after which the fleas were collected and fixed for electron microscopy studies. As controls, untreated and unfed fleas were taken and fixed for electron microscopy, as were fleas that had sucked once on an untreated dog.

In vitro

Approximately 100 adult and larval cat fleas were placed separately on the surface of filter papers inside plastic petri dishes. The filter papers had previously been impregnated with 500 µml of a 4–8% aqueous imidacloprid solution. Fleas were added after the filter paper had been air-dried for approximately 2 h or were covered with shaved hairs from imidacloprid-treated dogs. The behavior of the flea stages was observed with the aid of a stereomicroscope and a video camera. At 15 min after exposure the flea stages were taken out of the plastic dishes and fixed for electron microscopy. Adult and larval fleas were kept on filter paper (with and without water impregration) as controls. They were fixed for light and electron microscopy after 24 h.

Light and electron microscopy

Treated and untreated flea stages were dissected into two pieces (for better penetration of fluids) and immediately fixed in cold 5% glutaraldehyde (v/v) in 0.1 *M* sodium cacodylate buffer (pH 7.2). The osmolarity of the solution was adjusted to 300 mosmol with sucrose. The samples were postfixed for 2 h in 2% OsO_4 and dehydrated in graded cooled (0 °C) acetone. Finally, the fleas were embedded in ERL (epoxy resin of low viscosity) according to Spurr (1969). Ultrathin sections were stained with lead citrate and uranyl acetate (Reynolds 1963) and studied in a Zeiss EM S2 transmission electron microscope. Semithin sections were cut on a Reichert OMU 3 ultramicrotome, placed on gelatin-covered glass slides, and colored for 2.5 min with an aqueous solution of 4% toluidine blue and 4% malachite green on a heated plate. After being carefully rinsed in distilled water the sections were exposed for 15–20 min to a 4% aqueous basic pararosaniline chloride solution. Following further careful rinsing the sections were ready for light-microscope examination.

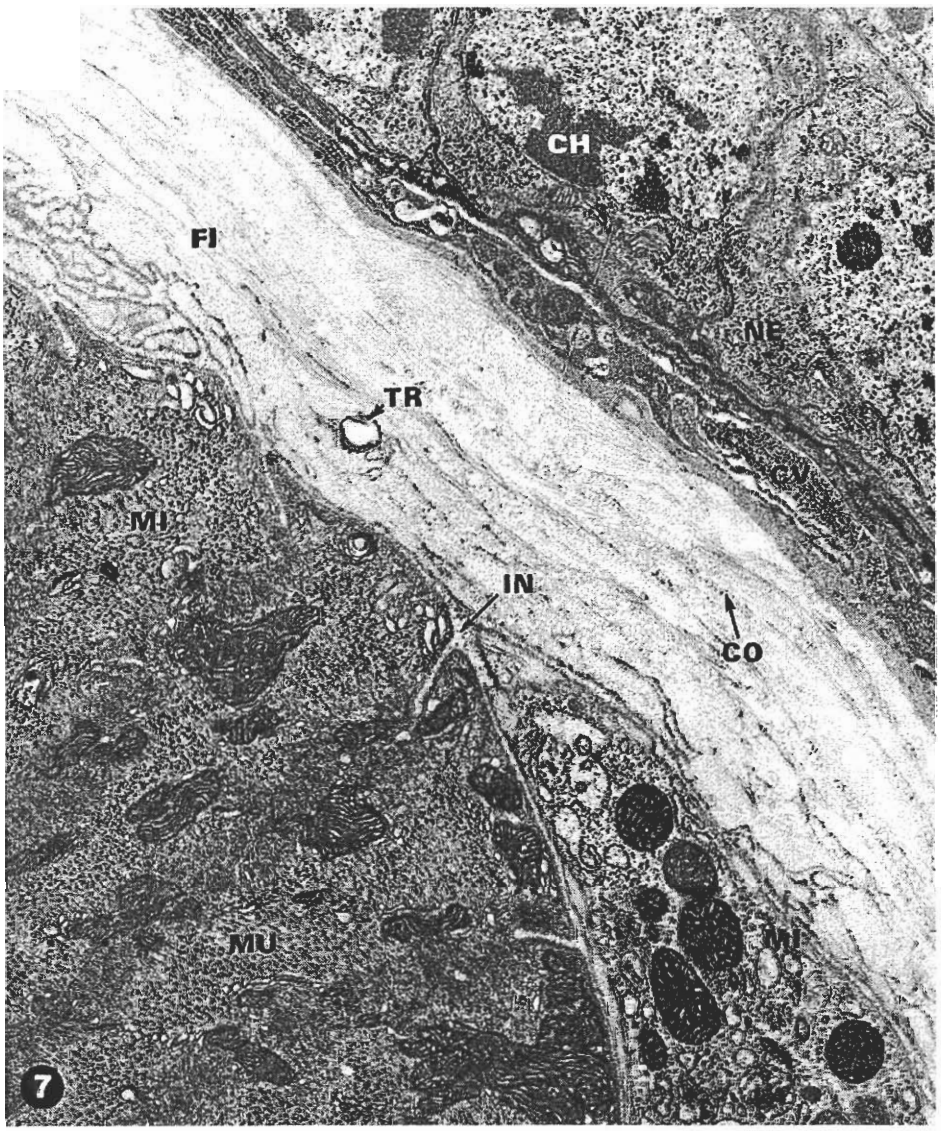


Fig. 7 Transmission electron micrograph (*TEM*) of a section through the head of an untreated adult control cat flea, showing the nerve cells of the subesophageal ganglion being surrounded by a thick lamellar layer of connective tissue and an adjacent strand of muscle fiber. ×50,000

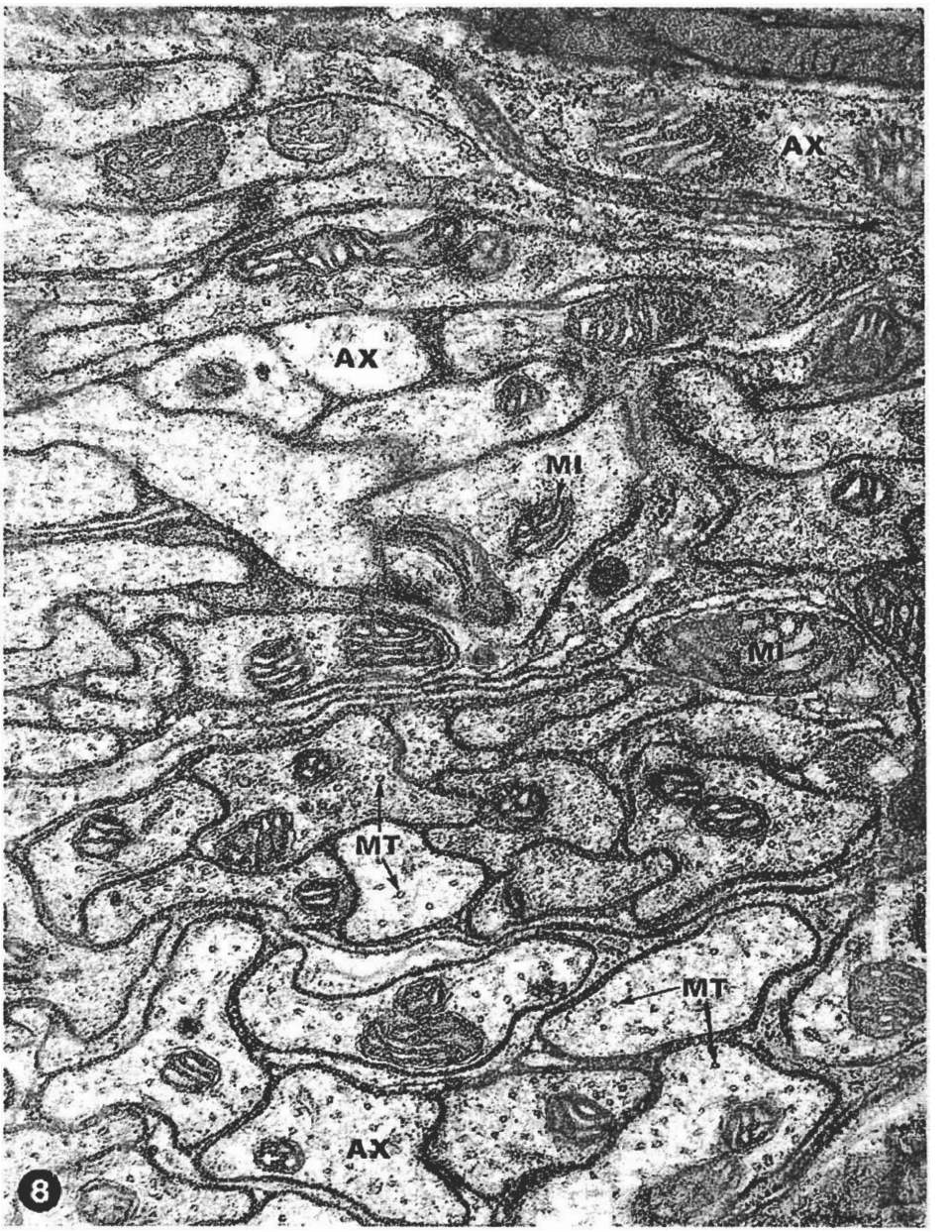


Fig. 8 TEM of a section through the neuropil of the brain of an untreated adult cat flea. Note that the cross-sectioned axons (*AX*) show typical features: intact mitochondria (*MI*) and microtubuli (*MT*). ×50,000

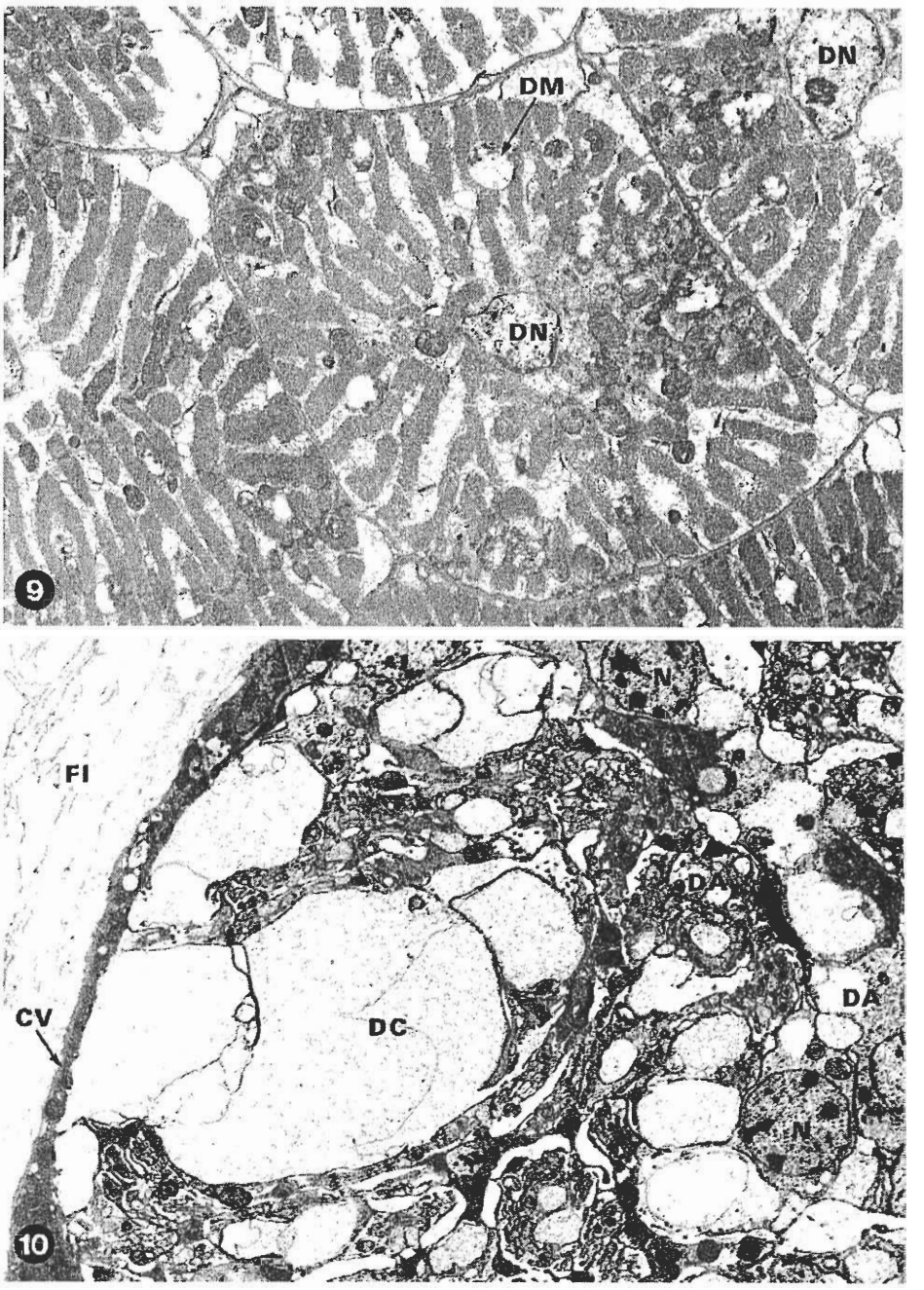
Video observations

Video observation was conducted using an Olympus SZH-10 stereomicroscope with a Sony (CDC-Iris/RGB) video camera and/or by a Panasonic hand camera. In all cases, S-VHS tapes were used.

Results

In vivo experiments

The experiments were repeated three times. For each experiment a dog received the calculated dose of imidacloprid (10 mg/kg) in a single "spot-on" on the neck. After 7 days a circular region on the dog's lateral side was shaved, and 100 unfed adult fleas were released onto this hairless skin region and covered by a plastic petri dish. Immediately after exposure the fleas attempted to feed on the dog. However, after 3–5 min, most of the fleas stopped their feeding activity and sought shelter at the periphery of the hairless zone. Their initial movements were slow, finally stopping, and rhythmic trembling of the legs and initiated rhythmic pumping movements of the abdomen were observed. At 10–25 min after the first occurrence of the trembling and pumping movements the fleas exhibited no activity and were apparently dead; thus, by 1 h after the first exposure of the fleas to the hairless skin region, all fleas had died.



Figs. 9, 10 TEMs of sections through an adult flea that had been exposed for 1 h to the shaved skin of an imidacloprid-treated dog. Note the clear degeneration at the level of the cross-sectioned muscles (**Fig. 9**) and along the periphery of the subesophageal ganglion. ×25,000

On morphological examination of these fleas, significant changes were seen in comparison with the untreated controls (Figs. 4, 7, 8). Initial damage occurred at the base of the fleas' "brain," i.e., along the subesophageal ganglion, which is fused with the hyperesophageal ganglion to form the anterior nervous system. This completely surrounds the esophagus (Figs. 2, 9, 10). In light microscopy these changes became visible in treated fleas as lightening of the zone of neurons (peripheral zone with the nuclei) and in the central region of the neuropil. The axons neighboring the esophagus showed no change (Fig. 2). Electron microscope observations showed that the cell bodies of the neurons located below the thick neural lamella and an inner cellular layer of electron-dense cells had disruption of the cytoplasm, vacuolization of most mitochondria, swelling of the perinuclear space, and degradation of the nuclear contents (Fig. 10). Control specimens fixed by the same method showed no degradation (Figs. 7, 8). The central region of the brain – the neuropil – was characterized by heavy vacuolization, along with the glial cells (Fig. 10). In the axons of this region the microtubules disappeared – in some axons, completely, and in others, partially – and many mitochondria became vacuolized.

Similar types of degeneration were seen in the thoracic ganglia as well (Fig. 5). As the first step of degeneration the striated muscle cells close to the ganglia and/or nerve bundles showed vacuolization of the mitochondria, followed by a slight and then intense separation of the single muscle fibers that were arranged in a ray-like fashion around the centrally situated nucleus. The latter were also destroyed (Figs. 6, 9). Whereas the sarcomeres remained intact with clearly visible Z-lines and myosin/actin interaction, the different invaginating tubular systems of the muscle cells were highly enlarged. Glycogen granules were absent inside these damaged cells, indicating irreversible degradation. In contrast, other cell types of the flea (i.e., intestinal cells or other cells belonging to the epidermal, respiratory, and sexual organs) showed no degeneration. Thus, the alterations caused by the imidacloprid treatment were confined to the ganglia and radiating muscles (Figs. 9, 10). The controls showed no such damage and were photographed in a highly functional state (Figs. 4, 7, 8).

Unfed fleas applied to skin from which the lipid layer had been removed with alcohol started feeding immediately. They engorged blood in the same way and, apparently, in the same amount as did the fleas on untreated dogs. They did not display trembling or pumping movements and remained mobile with normal displacing movements. After 1 h, none of the fleas had died. Examination of the fine structure of the muscle and nerves of these fleas revealed slight damage in some mitochondria of the nerve and muscle cells, but none was seen in the nuclei or in the arrangement of the muscular fibers (Fig. 11). In all cases, glycogen granules remained visible in the muscle cells. The number of typical microtubules and synaptical vesicles remained unchanged in the nerve axons (Fig. 11).

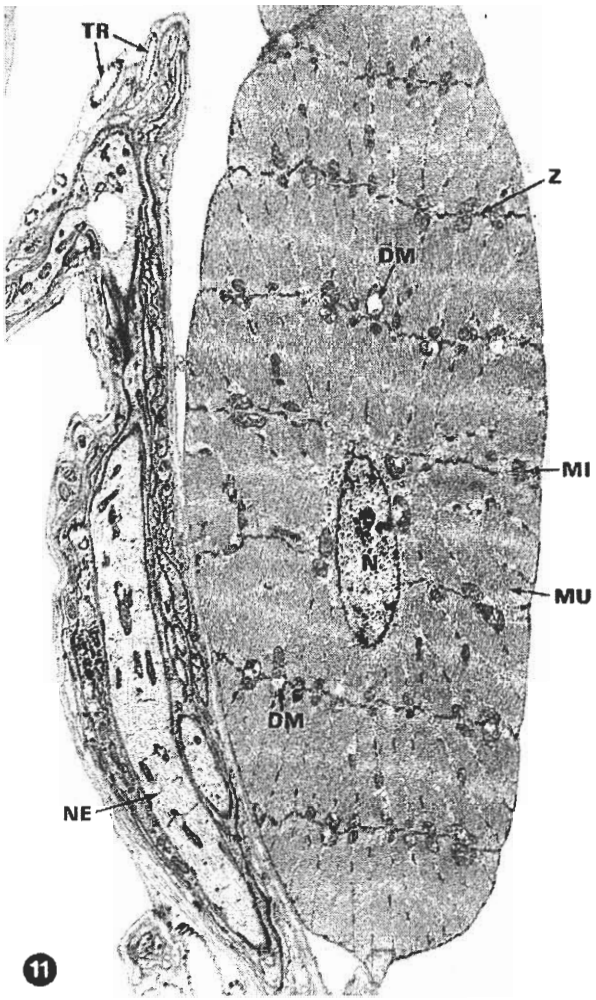


Fig. 11 TEM of a cross section through a muscle fiber and an adjacent nerve fiber (cut longitudinally) in an adult flea that had been exposed to the naked skin of a treated dog after defatting of that region with alcohol. Only slight damage is visible along some of the mitochondria (*DM*, *arrows*). ×25,000

In vitro experiments

Adult fleas

Approximately 100 unfed adult fleas were placed onto air-dried filter papers that had previously been impregnated with an aqueous solution of imidacloprid. At 5–10 min after application, some fleas ceased forward movements and began to exhibit trembling and pumping movements as seen in the in vivo experiments. After 20 min the fleas showing this behavior were incapable of leaving their position. When we studied sections of these fleas with the aid of the light and electron microscope it was obvious that they had incurred the same types of damage seen following in vivo incubation (Fig. 12). The degree and intensity of the cellular damage was related to the duration of exposure. Thus, the degeneration was less severe in the early phase, i.e., during the phase of trembling, than after 1 h, when all movements had ceased. The controls, which were kept on dry or wet filter papers, showed normal behavior and no morphological change after 2 days.

A second type of in vitro experiment was conducted that involved the mixing in a petri dish of 100 unfed adult fleas with hair from an imidacloprid-treated dog. At 1 h after the commencement of incubation, many of the fleas started trembling. At 2–3 h after the onset of trembling, 50% of the fleas were dead, whereas the rest remained active. At 15 h thereafter, 90% of the fleas were dead and the remainder showed the above-mentioned trembling movements. After another 8 h, all fleas in the petri dishes were dead. The control fleas on dry filter paper or on hair from the untreated control dog showed normal activity and behavior for up to 3 days of incubation.

Flea larvae

About 100 third-stage flea larvae (Fig. 13) were placed on dry filter paper that had previously been impregnated

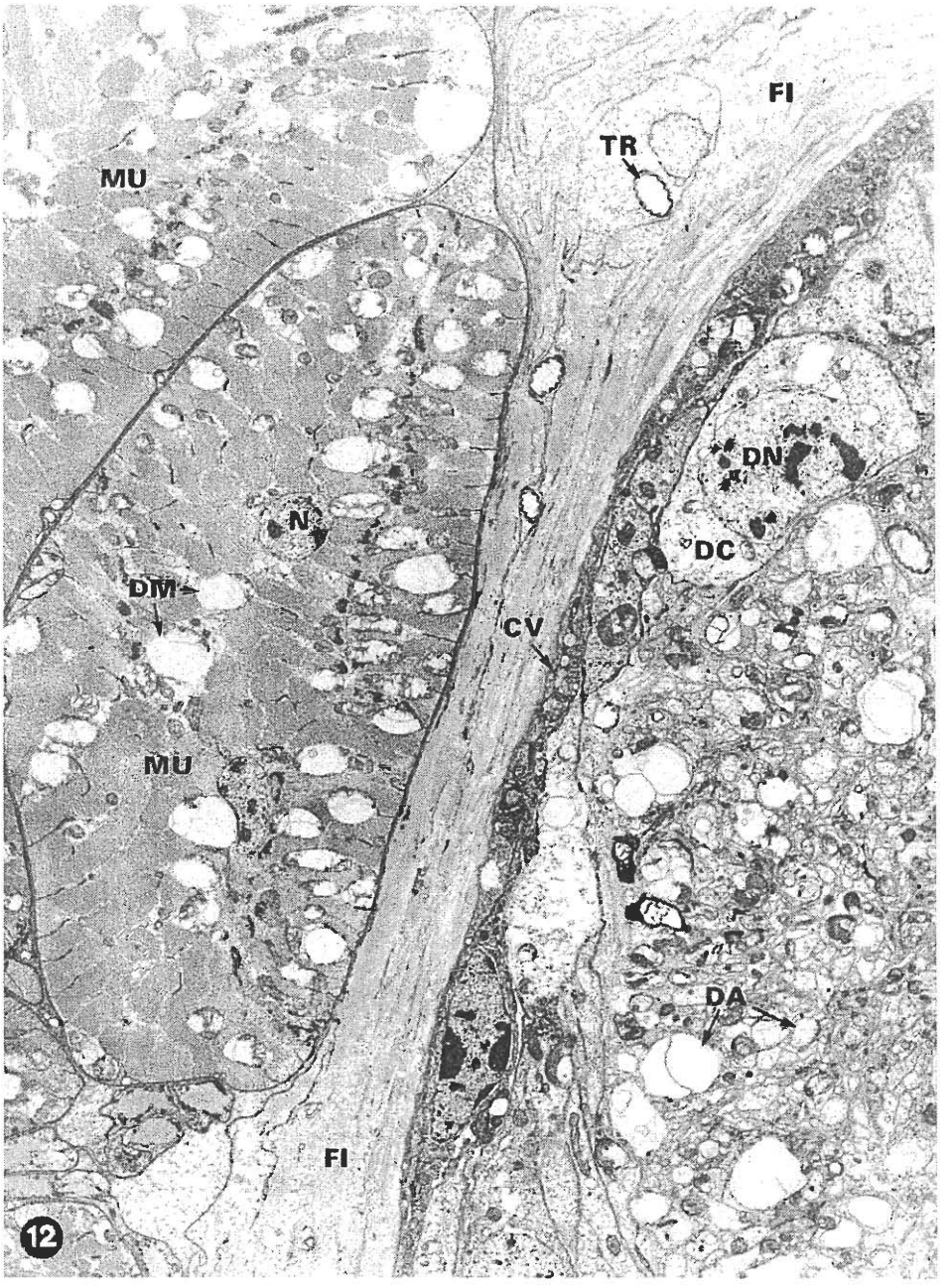


Fig. 12 TEM of a section through an adult cat flea that had been exposed in vitro for 1 h to imidacloprid-impregnated filter paper. Note the extensive damage at the level of the muscle fibers and of the subesophageal ganglion. Most of the mitochondria and many axons were vacuolized (*arrows*). ×25,000

with an aqueous solution of imidacloprid (experiment 1) or were added to shaved hairs from the imidacloprid-treated dog (experiment 2). In both cases the results were the same. At 15 min after the commencement of experiment 1 the larvae became motionless. However, their intestines showed pulsing movements for up to another hour. About 2 h later, all larvae were dead. The larvae that came into contact with the hairs of the imidacloprid-treated dog (experiment 2) showed the same symptoms as did the larvae in experiment 1; however, the effects took longer to be observed and several larvae survived for up to 6 h. The ultrastructure of these in vitro-treated larvae showed damage at the level of mitochondria (in nerve and muscle cells), leading to swelling and disruption (Figs. 16–18), whereas mitochondria in other cell types (e.g., in the epidermis) showed no alteration. Several axons in the ventral nerve chord as well as other nerve strands exhibited damage. Then the spaces between the fibrillar bundles in the striated muscle cells became enlarged and the glycogen granules gradually disappeared (Fig. 1). The degeneration was visible only in places and did not occur to the same extent in the whole nervous or muscle system (Figs. 17, 18). Furthermore, the degree and intensity of

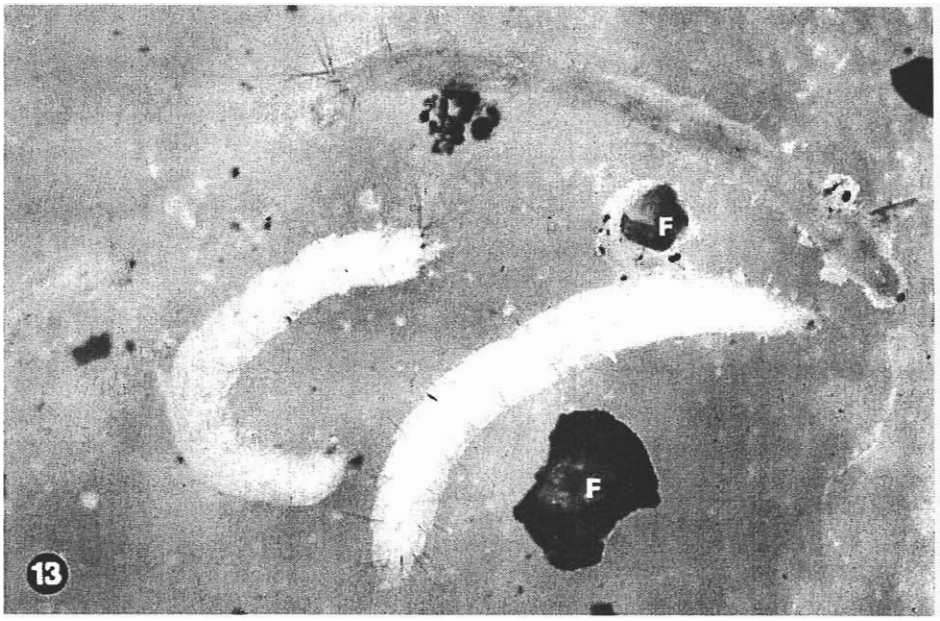


Fig. 13 Light micrograph of several flea larvae at different stages of development. ×20

the damage was much less intense than that of the damage visible in the tissues of imidacloprid-treated adult fleas. The control larvae maintained on normal food, filter paper, or hair from the untreated dog showed normal behavior and motility for several days after the death of those that had come into contact with imidacloprid.

Discussion

Mode of uptake

Imidacloprid[2] is used worldwide in agriculture against a variety of insect pests on crops. When the seed or the whole plant is treated, insects feed on these plants and die within a short period (Oetting and Anderson 1990; Elbert et al. 1991; Bai and Lummis 1991; Chao et al. 1997; Nauen 1994). Whereas plant-sucking aphids take up the insecticide in their food, the present in vitro experiments using filter paper or hair that had been impregrated with imidacloprid emphasize that the compound does not have to be ingested to be effective, since neither the adult fleas nor the larvae had contact with dog blood. This finding corresponds to previous experiments using the "artificial dog" system (Fichtel and Ewald-Hamm, unpublished results). It can be concluded that uptake occurs via the smooth, nonsclerotized intersegmental membranes that are responsible for the insects' mobility (Figs. 5, 6). This seems reasonable because the lipophilic properties of imidacloprid render it incapable of passing through the sclerotized cuticle, and initial damage was seen in the ganglia close to the ventral body side (e.g., in subesophageal and thoracic ganglia). The killing effects of the fatty hair from imidacloprid-treated dogs on larval and adult flea stages indicate that the compound is included in the surface lipid layer, which is produced by sebaceous glands and spreads over the body surface. Since this lipid layer is always present, imidacloprid remains available for a prolonged period (Hopkins et al. 1996; Hanssen et al. 1999; Mehlhorn et al. 1999) The location of the compound in the lipid layer reduces the likelihood of its removal during swimming or by rain.

Effects on the flea

The experiments described in the present paper clearly demonstrate the larvicidal and adulticidal activity of imidacloprid. Both stages are sensitive to the drug, and after contact they react in a similar fashion: they stop their jumping or (respectively) crawling movements and display the onset of rhythmic trembling of the legs and the body. This nonreversible phenomenon finally leads to the death of both flea stages. These easily visible effects correspond to the finding that imidacloprid blocks the postsynaptical nicotinic acetylcholine receptors (Abbink 1991). The latter are normally stimulated by acetylcholine that is excreted into the synaptic gap. These receptors initiate the opening of channels in the membrane to let Na^+ flow into the cell. This leads to a depolarization of the terminal plate and induces the activation of an action potential. The latter causes the release of Ca^{2+} from vesicles and thus results in contraction of the myosin/actin complex of the sarcomeres. In normal cases the acetylcholine has a brief connection to the receptor, is subsequently released, and is rapidly

[2] Trademarks are Admire®, Confidor®, Gaucho®, and Provado®

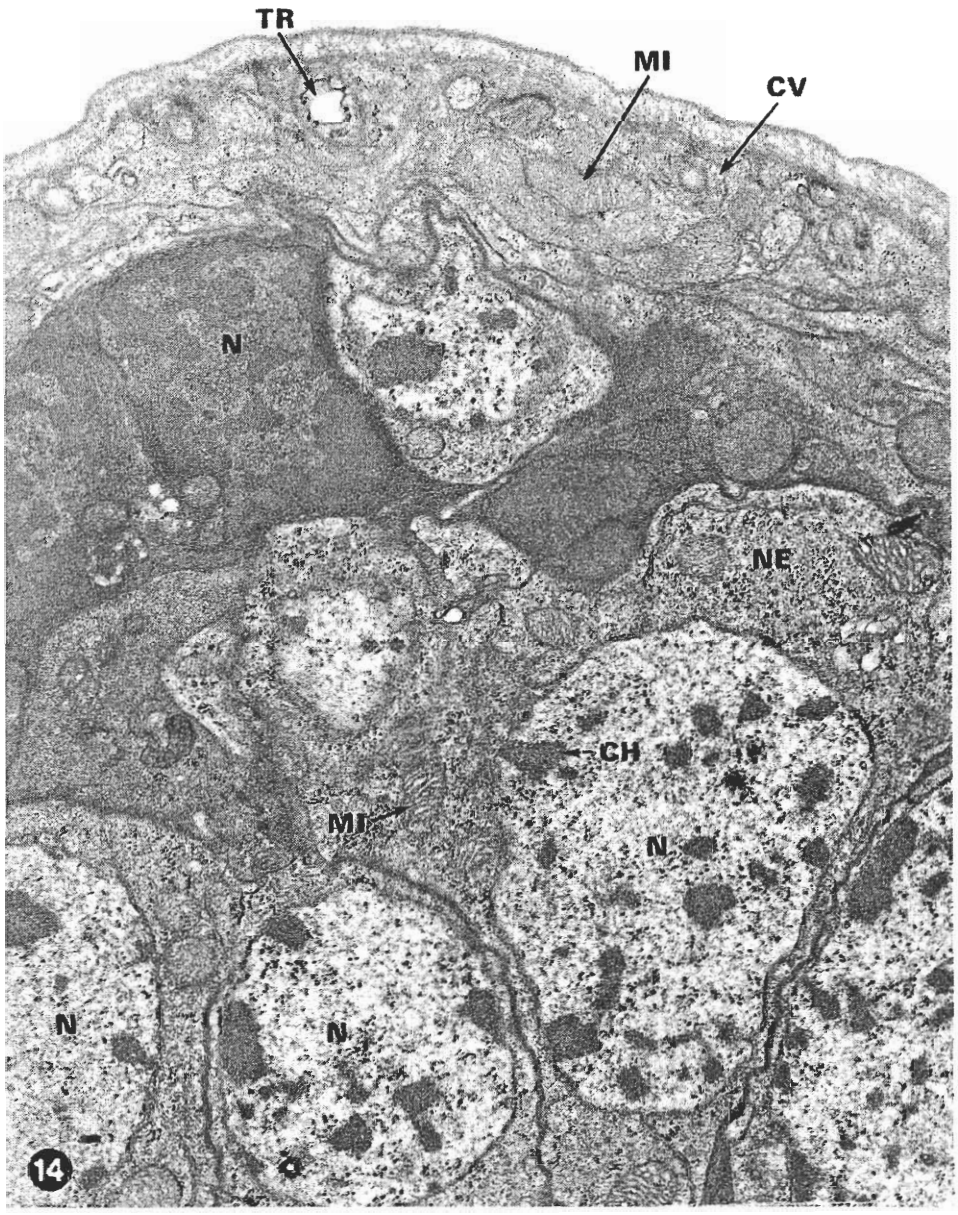
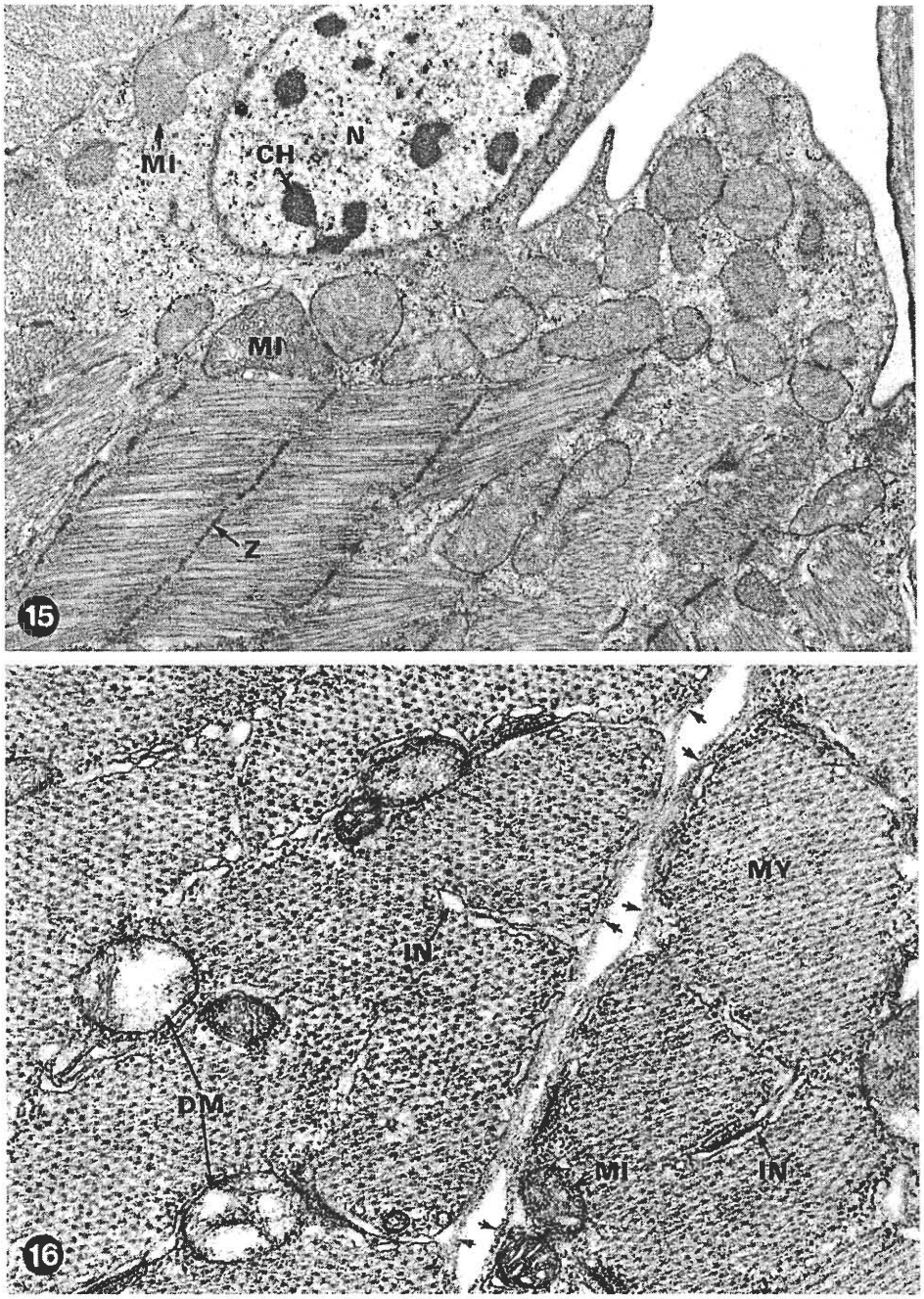


Fig. 14 TEM of a section through the periphery of the anterior nerve chord of an untreated larva (control). Note the intact layer of nerve cells with their large nuclei; some nerve cells appear electron-dense. ×50,000

hydrolyzed by a membrane-bound cholinesterase. In the case of imidacloprid the binding of the compound and the receptors is stronger; hence, a constant depolarization of the membrane occurs, inducing a tetanus of the activated muscle cell. This mode of action corresponds to the structural findings described herein, since the observed degeneration mainly involved an overall destruction of the mitochondria, damage to the nerve cells, and disintegration of the muscle. Imidacloprid initiates a constant depolarization of the nerves, which is followed by a constant activation of the muscles until the cellular energy systems (mitochondria, glycogen) are depleted and the motile proteins are destroyed.

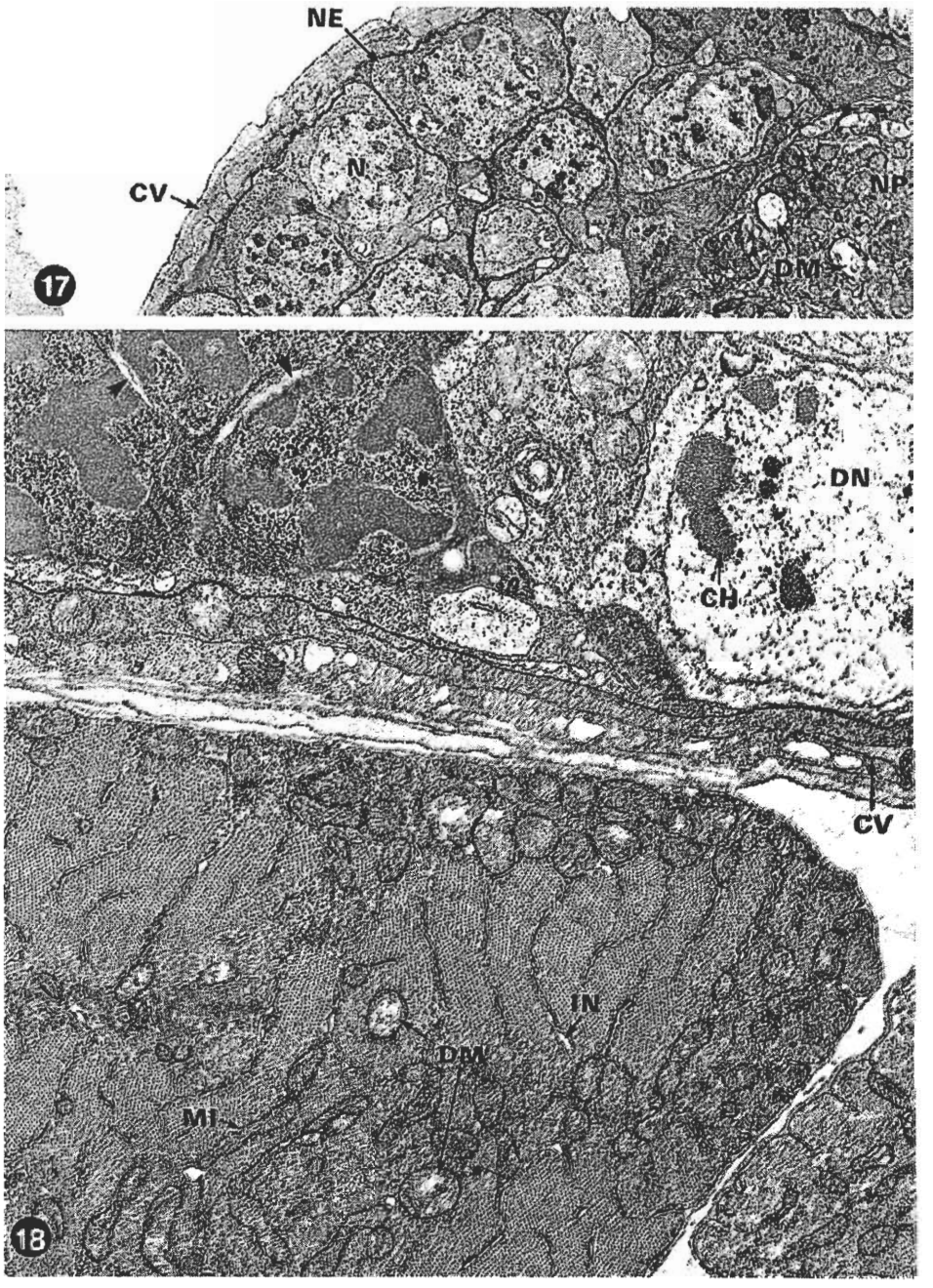
Originally it was thought that nicotinic acetylcholine receptors in insects were situated exclusively in ganglia (Breer and Sattelle 1987). This was supported by observations that the green plant-louse *Myzus persicae* remains mobile for a prolonged period after feeding on an imidacloprid-treated leaf (Nauen 1994). However, the finding of stationary immobility and intense continuous tetanus-like trembling in fleas suggests that there may be additional blocking processes, generally at the connections between nerves and muscles. The selective toxicity of imidacloprid for insects as compared with mammals, whose sensitivity is 1000-fold lower, has recently been explained by the finding of different subunits of the nicotinic acetylcholine receptor (Latli et al.



Figs. 15, 16 TEM of sections through the muscle fibers of flea larvae. **Fig. 15** Untreated control. ×50,000. **Fig. 16** Treated larvae (exposed to imidacloprid-impregnated filter paper); note the disruption of muscle bundles in the cell and the disintegration of mitochondria (*MI*). ×75,000

1997; Matsuda et al. 1998; Schulz et al. 1998). That imidacloprid does not significantly penetrate the skin further enhances its safety in vertebrates; thus, its safety factor is more than 10,000 for cutaneous application (Kagabu 1997).

In conclusion, imidacloprid was shown to act as both a larvicide and an adulticide in studies on cat fleas. Due to its probable main uptake by the flea through the nonsclerotized intersegmental membranes it rapidly reaches the site of action: the postsynaptic membrane. There, the irreversible blocking of the nACh receptors leads to a lethal hyperactivity of the nerves and muscles of the insect. The inclusion of imidacloprid in the lipid layer of the skin surface and the hair prevents the compound from being washed off (by rain or during swimming). It also reduces the possibility of skin penetration. As adult and larval fleas are rapidly killed upon contract with imidacloprid, whether in impregnated



Figs. 17, 18 TEM of sections through flea larvae that had been exposed to imidacloprid-impregnated filter paper for 15 min. **Fig. 17** Outer surface of the esophageal ganglion; note the degeneration at several axons (*arrows*). ×25,000. **Fig. 18** Section through the contact zone between a ganglion (*above*) and a muscle fiber (*below*); note that in the ganglion the nuclei of the nerve cells show swellings in the perinuclear space (*arrowheads*). Nuclear disintegration is visible as lightened areas. At the level of the muscle cell, some of the mitochondria are swollen (*double arrows*), and the invaginated tubular systems are enlarged. ×45,000

hairs or in filter papers, the compound has the potential to prevent the establishment of a flea population in households.

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