Tissue damage after acute intoxication by polychlorinated biphenyls (PCBs) in cockroaches Blattella germanica

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It is common knowledge that polychlorinated biphenyls (PCBs) represent a serious threat to the health of both vertebrates and invertebrates. As far as the former are concerned, especially as regards human beings, a broad literature describes the direct and indirect effects induced by the PCBs on their systems and organs. Among invertebrates, the information available is mostly related to arthropods and is, however, very scarce. The aim of this work was to evaluate the effects of polychlorinated biphenyls (PCBs) on tissues and organs of individuals belonging to a species of Blattaria *(Blattella germanica)* treated with various doses of this toxic material. The pathologies found became more serious as the dosage increased and were present throughout the entire digestive system, in the fat body and in the male gonads: in these areas cell and tissue breakdown and severely damaged spermiogenesis were observed. In particular, the testicles, Malpighian tubules and fat body accumulated an amorphous basophilic PAS-positive substance. Furthermore, the NOS-dependent NADPH diaphorase activity pattern in the retina and optic lobes was more evident in the treated than in the control insects.

Key words: polychlorinated biphenyls, cockroaches, *Blattella germanica*, tissue pathology.

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Several chemical agents pose significant and global threats to human and wildlife health (WHO, 1993). Some of these, such as polychlorinated biphenyls (PCBs), remain in the environment, become dispersed for thousands of miles, and bioaccumulate within food chains (Vallack, 1998; Fisher, 1999). They are ubiquitous environmental pollutants resulting from intensive industrial use and inadequate disposal over the past decades (Erickson, 1986).

It has been stated that PCBs, by binding to the cytosolic aryl hydrocarbon (Ah) receptor, are responsible for pathologies concerning the reproductive system of vertebrates, damage to the immunological system, teratogenesis, and carcinogenesis (Okey *et al.*, 1994; Safe, 1994). On the other hand, recent studies indicated that neurotoxic effects of PCBs might not be mediated through the Ah receptor (Schantz, 1996; Seegal, 1996).

Knowledge of the effects of PCBs on insects derives basically from research performed on *Musca domestica* by Tehseen *et al.* (1992) and by Saghir and Hansen (1994, 1995, 1999), who studied the survival parameters of insects treated with PCBs and the distribution of these substances within tissues. The work of Li and McKe (1992) on the house cricket has shown that the concentration and the nature of the congeners of PCBs found in tissues are directly correlated to their lipid content. In this work, we have tested the pathological effect of PCBs on organs and tissues of a species of Blattaria *(Blattella germanica)*. This particular species, because of its being the frequent prey of vertebrates, and its interaction with the anthropical community,. is probably responsible for the diffusion of the PCBs throughout the biome.

Materials and Methods

The tests were performed on *Blattella germanica* L. (Blattaria, Blattellidae). A 5 ml DCMA PCB mixture - Supelco vial contained 1.3 μg PCBs/5 μL

n-hexane. The ingredients per 5 mL vial were: 100 μg/mL 2-chlorobiphenyl (MCB), 100 μg/mL 3,3′-dichlorobiphenyl (DCB), 10 μg/mL 2,4,5-trichlorobiphenyl (TCB), 10 μg/mL 2,2′,4,4′-tetrachlorobiphenyl (QCB), 10 μg/mL 2,3′,4,5′,6-pentachlorobiphenyl (PCB), 10 μg/mL 2,2′,3,3′,6,6′-hexachlorobiphenyl (HCB), 5 μg/mL 2,2′,3,4,5,5′6-heptachlorobiphenyl (ECB), 5 μg/mL 2,2′,3,3′,4,4′,5,5′-octachlorobiphenyl (OCB), 5 μg/mL 2,2′,3,3′,4,4′5,5′6-nonachlorobiphenyl (NCB), 5 μg/mL 2,2′,3,3′,4,4′,5,5′6,6′-decachlorobiphenyl (10CB). A 5 mL vial of DCMA PCB mixture was concentrated to 50 μL by blowing a 60 mL/min nitrogen current and then taken to 5 mL with triolein (Sigma).

Two hundred last instar (6th) male nymphs were divided into five groups of forty individuals each; the specimens of each group were injected with either 0.5, 1.0, 3.0, or 5.0 μL of triolein containing 0.26 μg PCBs/μL, such that the groups received respectively, 0.13, 0.26, 0.78, 1.3 μg of PCBs; the controls were injected with 5.0 μL of triolein.

The doses of PCBs were administered by a *Cell Tram Vario* Eppendorf apparatus and a *Bactotrip* Eppendorf needle. The nymphs were anaesthetized with CO2 and injected through the non-sclerotized cuticle, on the side of the epimeron of the third thoracic segment. This way the needle could penetrate within the coxa.

Survival rates were checked daily throughout the 20-day experimental period. The data were tested by Life Table Analysis, Wilcoxon statistics, and pairwise comparisons.

Control and treated nymphs were collected between the 15th and the 20th day after injection, anaesthetized with CO2, and dissected in Yeager's solution (Yeager, 1939). To remove the ventral nerve cord, brain, gonads, intestine, Malpighian tubules and abdominal fat bodies, the tissues were fixed in methanol: acetic acid (3:1) for 1h, dehydrated in absolute ethanol for 2 hrs, embedded in methacrylate ester, sliced into 3-5 μm sections and stained with 1% toluidine blue or tested for the PAS and PAS-diastase reaction.

The activity of NOS (nitric oxide synthase)-dependent NADPH diaphorase was evaluated histochemically on the retina and brain. The tissues were prepared by removing partially the cuticle of the *frons* and of the *vertex* up to the occipital suture in order to expose the brain and the optic lobes. The heads were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.35, for 2 h. After being washed in the same buffer for 30 min, they were stored in a cryoprotective 25% sucrose-phosphate buffer overnight at 4°C and then frozen in liquid nitrogen. On the frontal plane, serial 10 μm cryostatic sections were prepared. The sections were incubated for 1 h at 37°C in the dark in a 0.1 M phosphate buffer solution, pH 7.35, containing 15% (w/v) polyvinyl alcohol, 0.5 mM β-NADPH (Sigma, MO, USA), 0.2% Triton-100 and 5 mM nitro blue tetrazolium (Sigma) as reported by Conforti et al (1999). After incubation, the sections were rinsed in a 0.1 M phosphate buffer, pH 7.35, mounted on glycerine-jelly and observed through a Zeiss Axioskop microscope.

Results

The survival rate of the cockroaches did not differ among the controls and the specimens treated respectively with 0.13 μg and 0.26 μg of PCBs; the cockroaches treated with 0.78 μg and 1.30 μg showed significantly lower survival rates than each of the other categories (Figure 1). The low survival rate of cockroaches in the 0.78 μg treatment was due mainly to deaths of recently metamorphosized adults; in the 1.30 μg treatment most deaths occurred in nymphs prior to their metamorphosis.

The severity of histological abnormalities observed increased as the amount of PCBs injected was increased. Insects subject to treatment with 0.13 μg of PCBs showed mild histological abnormalities and those treated with 0.26 μg of PCBs showed mild to moderate alterations of organs and tissues. Nymphs treated with 0.78 μg and 1.3 μg of PCBs showed severe, diffuse structural damage independently of their survival span, with occasional mycotic colonizations. Deep histopathological alterations were observed in the alimentary tract, in the male gonads and in the fat body, while the cells of the nervous system seemed only mildly altered; the ovary did not show structural alterations. Control insects treated only with triolein showed very subtle abnormalities.

The foregut of the controls and of the insects treated with 0.13 μg of PCBs did not show histological abnormalities, whereas the foregut of those treated with 0.26 μg of PCBs was mildly altered. The epithelial cells of their foregut were occasionally vacuolated, but the nuclei did not show significant abnormalities. Stronger alterations character-

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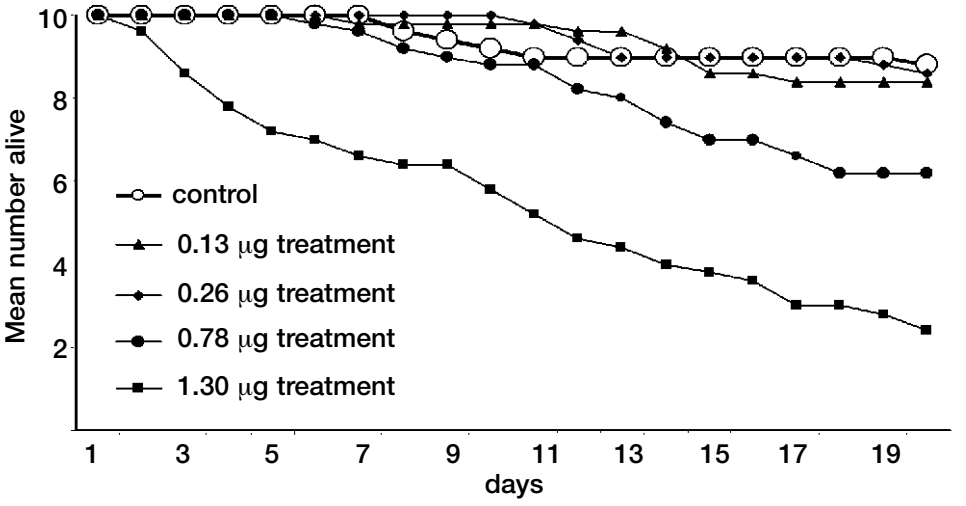

Figure 1. Survival of treated and control cockroaches. The mean values were calculated for 5 independent experiments, each one involving 10 insects per each treatment and control. Differences were highly significant ($p<0.01$) for the difference between 0.78 μg PCBs treatment group vs each of the other treatments and $p<0.0001$ for the difference between the 1.30μg PCBs treatment group and the other treatments; all other differences were not significant). Wilcoxon pairwise comparison test.

ized the foregut of the nymphs treated with 0.78 μg of PCBs: focal epithelial cell loss, nuclear enlargement or pycnosis were observed; however the pseudopapillary projections of the epithelium in the lumen, and the cuticular coat, were maintained. Extensive alterations, with widespread epithelial damage and structural degeneration were observed in the foregut of insects treated with 1.3 μg of PCBs. A deep dismantling of the epithelial layer with a partial cellular loss, massive cellular vacuolization, and nuclear damage and degeneration were observed. The most striking alteration was the protrusion into the intestinal lumen of the cuticular coat (Figure 2 A, B). Salivary glands were hyperplasic, with dilated lumina frequently containing degenerated nuclei (not shown).

The midgut of the control insects and of those treated with 0.13 μg of PCBs did not show histological abnormalities. The 0.26 μg dose produced a partial derangement of the nuclear linear array columnar cells; cytoplasmatic vacuoles in columnar cells and pycnotic nuclei in the crypts were present; the lumen contained a thick secretion, that comprised degenerated nuclei from the foregut epithelium. The brush border was only focally present, and the peritrophic membrane was lost. In the midgut of nymphs injected with 0.78 μg of PCBs, occasional columnar cells contained infranuclear vacuoles, and the nuclear arrangement was focally discomposed. Rare pycnotic nuclei were present in the crypts. In the midgut of nymphs treated with 1.3 μg of PCBs an abnormal secretion separated the epithelium from the basement membrane. Occasionally crypts were displaced and lay free in the secretion. The epithelial layer was also frequently interrupted or infolded. The nuclear array was only minimally disorganized but extensive supra- and infranuclear vacuolarization testified to cellular damage. Pycnosis was observed in the crypts; the brush border was focally altered, while the perithrophic membrane, if present, was partially separated from the epithelial surface (Figure 2 C, D).

The hindgut of controls and nymphs treated with 0.13 μg and 0.26 μg of PCBs showed only mild histological abnormalities of the epithelial cells; in nymphs treated with 0.78 μg and 1.3 μg of PCBs, it was severely affected: the epithelial layer was strongly discomposed, vacuolated, lacking in peritrophic membrane and infected by wide mycoses that caused a focal reaction of encapsulation of the fungal parasites often expanding into the layer that divides the epithelium from the muscles (Figure 2 E, F).

The Malpighian tubules in insects treated with 1.3 μg of PCBs showed severe nuclear damage with karyorrhexis, cytoplasmic vacuolization and nuclear displacement toward the lumen. Dramatic damage, with storage of amorphous-to-finely granular basophilic material within the cells and lumen was observed in some specimens. Most cells were

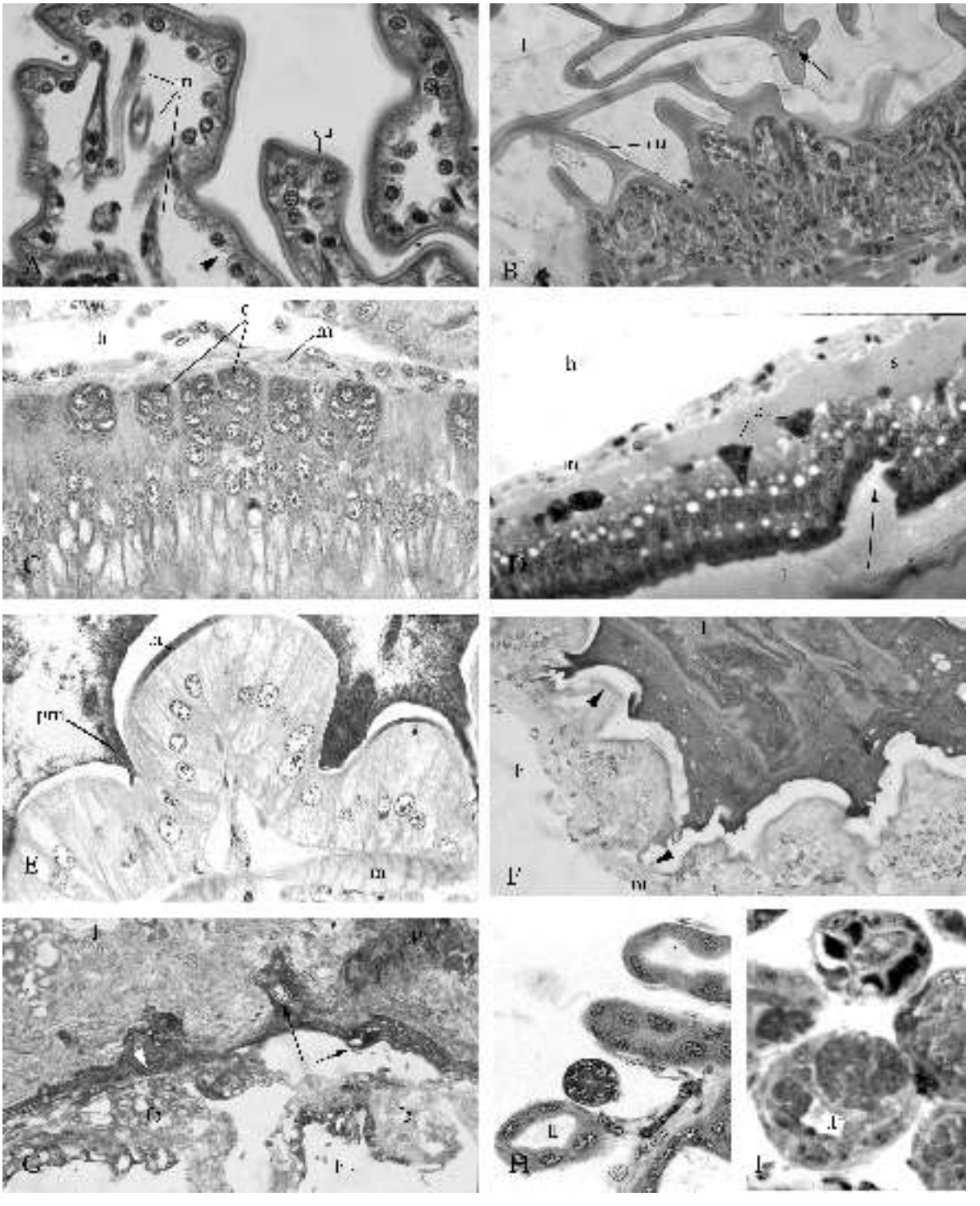

Figure 2. Intestine and Malpighian tubules sections of untreated and PCB treated nymphs. A, foregut of an untreated nymph: the peculiar *lace profile* (arrowhead) of the monolayer epidermis facing the muscular plane is evident (x500). B, foregut of a nymph treated with 1.3 μg of PCBs: cuticular digitations, among which some epithelial cells are observable (arrow); disorganization of the epithelium and wide intracellular gaps are visible (x200). C, midgut of an untreated nymph (x500). D, midgut of a nymph treated with 1.3 μg of PCBs: the crypts are often dispersed in the secretion between the muscular plane and the epithelium; gaps are visible between the secretive cells (arrow) (x500). E, rectal gland of an untreated nymph (x500). F, rectal gland of a nymph treated with 0.78 μg of PCBs: lyses of the cellular membrane and focal loss of the cuticular layer (arrowheads) are evident (x200). G, rectal gland of a nymph treated with 1.3 μg of PCBs: degeneration of the glandular cells and loss of the topographical ratio with the muscular plane (arrowhead); the encapsulation of the fungal structures appears evident (arrows) in the material generated by the immunocyte phenoloxidase system (x200). H, Malpighian tubules of an untreated nymph (x500). I, Malpighian tubules of a nymph treated with 1.3 μg of PCBs: numerous cells are engulfed with amorphous basophilic material visible also in the lumen (x200). c, crypt; cu, cuticle; fb, fat body; h, hemocoele; m, muscle; pm, peritrophic membrane; s, secretion; tl, lobular lumen.

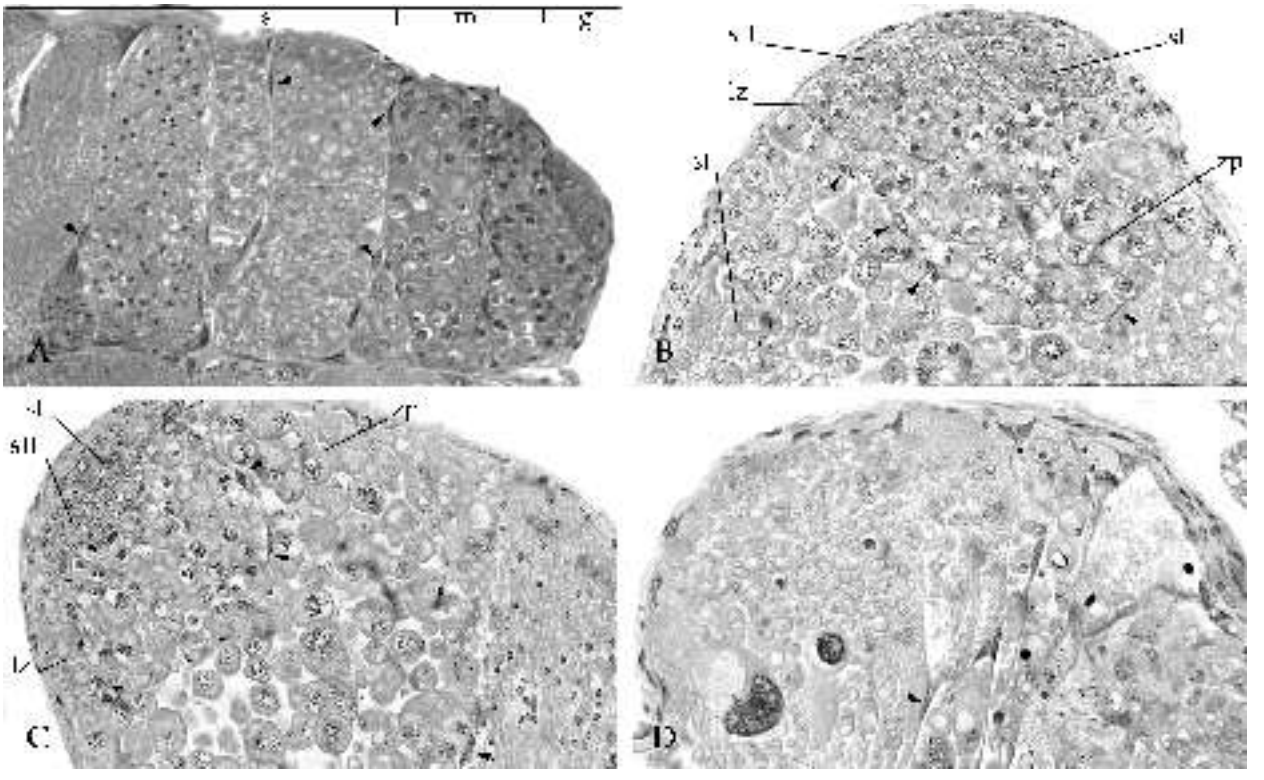

Figure 3. Longitudinal sections of the testicular lobes of Blattella germanica (x200). A, an untreated nymph. B, in nymphs treated with 0.13 μg of PCBs a marked difference in size among zigo-paticheni is visible; some cells are bi- or trinucleate; some nuclei of the theca are pycnotic. C, in nymphs treated with 0.26 μg of PCBs fusion of contiguous spermatocysts is observable. Along with the great difference in size of the zigo-pachiteni, spermatids in their initial phases of development are visible. D, in nymphs treated with 1.3 μg of PCBs a vast degeneration of all the spermatocysts including those containing spermatids are noticeable. The meiotic phases are not identifiable. It is possible to see numerous pycnotic nuclei in both somatic and germinal cells. g, gonadal area; lz, lepto-zigotene; m, meiotic area; sI, pimary speratogonia; sII, secondary spermatogonia; s, spermiohistogenetical area; st, spermatids; zp, zigo-pachitene; arrowheads, spermatocyst cells.

totally engulfed with this material and lost any cellular structure they had (Figure 2 H, I). Smaller amounts of the same material were observed in the connective cells outlying the midgut and the gonads and in the fat body cells. The above-mentioned material showed marked PAS-positiveness before and after diastase digestion.

The male germinal line of control insects did not show any abnormality. The testes of insects treated with 0.13 μg and 0.26 μg of PCBs showed asynchrony of meiotic phases, apparently generated by the fusion of contiguous spermatocysts; degenerated nuclei were seen among the spermatogonia; giant and multinucleated meiocytes were frequent; chromatids were often dispersed unevenly in enlarged nuclear spaces; a few thecae of lepto-zygotene nuclei were damaged, and chromatids entered the cytoplasm. Pycnotic nuclei were present in every spermatocyst. Spermatids and spermiocytes were substantially unaltered, along with the overall structure of the spermatocysts. Insects injected with 0.78 μg and 1.3 μg of PCBs showed a higher degree of asynchrony, with frequent abnormal anaphases; late spermatocysts were altered, containing giant and multinucleated cells, with irregular masses of amorphous, lightly basophilic material, among which more basophilic hyaline globules resembling nucleoplasm were present. Since spermatids and spermatocytes had almost completely disappeared, the said material could represent degenerated spermatids. The cellular boundaries of spermatocysts were discontinuous; sustentacular cell nuclei were markedly enlarged (Figure 3).

The fat bodies of the control insects did not reveal any pathological picture. In the specimens treated with a low concentration of PCBs, a few bacteriocytes showed ruptures and bacterial dispersion. A few adipocytes were also damaged and confluent empty spaces were formed. Nuclear degeneration was observed both in bacteriocytes and trophocytes. In insects treated with 0.78 μg of PCBs, all

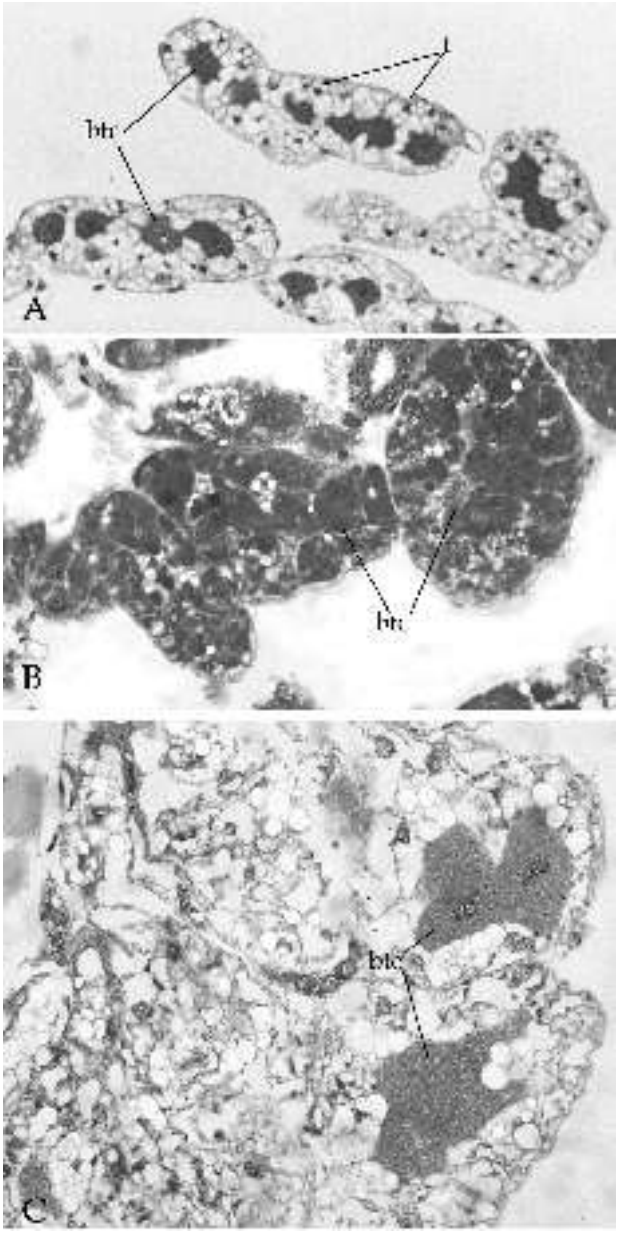

Figure 4. Sections of fat body lobes of Blattella germanica. A, an untreated nymph (x100). B, in nymphs treated with 0.78 μg of PCBs the cells of the fat body lobes appear engulfed with amorphous basophilic material PAS positive (x200). C, in nymphs treated with 1.3 μg of PCBs disintegration of cell components and bacteriocyte migration towards the surface of the lobes are observable. Some of these bacteriocytes are apparently fused (x500). btc, bacteriocytes; t, trophocyte.

cell types were almost completely engulfed by an amorphous basophilic material that reacted strongly to the PAS reaction, before and after diastase digestion. In the insects treated with 1.3 μg of PCBs, the structure of trophocytes and urocytes was lost and a few bacteriocytes migrated towards the lobe surface. Apparently the bacteriocytes underwent cytoplasmatic fusion. Nuclear degeneration was observed (Figure 4). The same substance above-described was observed in the connective cells outlining the midgut and the gonads, and in most of the cells of the Malpighian tubules. Many hemocytes stratified on the external surface of the lobes.

In control insects, the nervous system cells occasionally showed margination of the chromatin. Minimal abnormalities were seen with the lower dosage. The insects treated with 1.3 μg of PCBs had more severe nuclear alteration in large neurons. The chromatin was separated from the nuclear theca by an empty space that created a clear halo. Some of these nuclei showed karyorrhexis, and in others the chromatin was condensed. Small neurons seemed less damaged, with only few pycnotic nuclei. The nuclei of external sustentacular cells had shrunk and become hyperchromatic. The overall structure of the nervous system was preserved.

The general pattern of the NOS-dependent NADPH-diaphorase activity of the retina and the optical lobes of the brain was characterized by a deeper staining of the treated insects compared to the controls. Marked differences were visible in the neuropilar (proto- and deutocerebral) regions: in insects treated with 1.3 μg of PCBs, the mushroom bodies (calyx, stalk, Kenyon cell region) and the antennal lobes (neuropilus, glomeruli) showed much stronger reactivity than in the controls; only a few glial cells were strongly reactive. Fibers showing a regular pattern, thickened, and strongly labelled were visible within the retina and the neuropilus of the antennal lobes (Figure 5).

Discussion

The PCBs distribution in tissues varies widely (Saghir and Hansen, 1999); their action appears to affect dramatically different biological functions in all the invertebrate taxa studied; in fact, these substances are able to influence the functions related to the development, the metamorphosis and the longevity of the exposed species (Duke *et al.*, 1970; Sanders and Chandler, 1972; Nimmo *et al.*,1975; van Urk *et al.*, 1992; Tehseen *et al.*, 1992), as well as the functionality of the nervous system (Hansen *et al.*, 1974) and the endocrine/neuroendocrine system (der Besten *et al.*, 1991); moreover, they induce structural pathologies in tissues and organs (Nimmo *et al.*, 1975) and modifications of enzy-

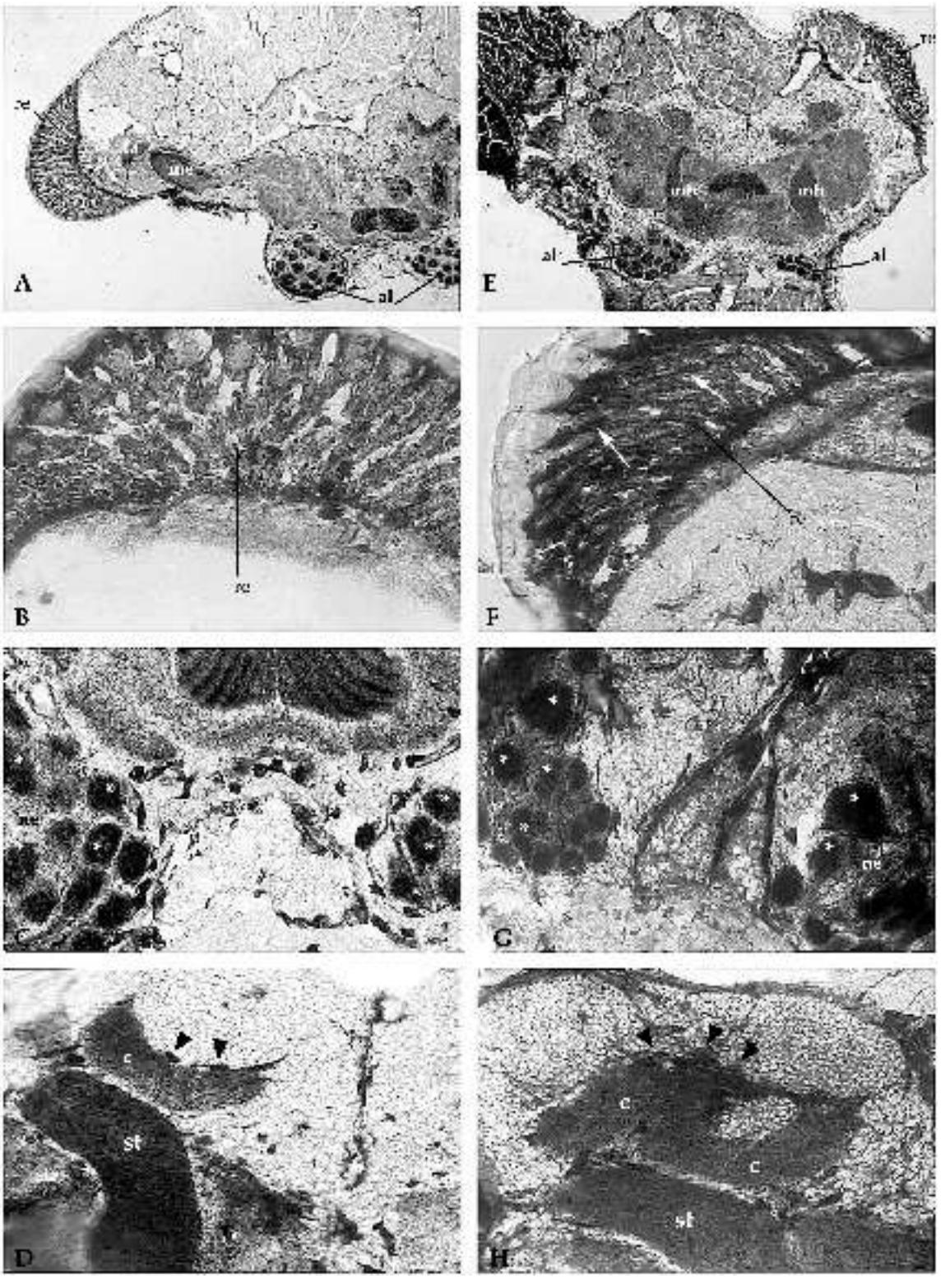

Figure 5. Distribution of NADPHd staining in the brain of Blattella germanica. A, B, C, D, brain frontal sections of control insects. A generally intense NADPHd staining is detectable throughout the brain. The staining is strong in the antennal lobes (al) [in particular in the glomeruli (asterisks), in the calices (c) and in the stalk (st) of mushroom bodies (mb)]. Labelling is also present in the two neuropilar areas of the optic lobes [lamina (la), medulla (me)], whereas the retina (re) is not stained (A, 10x; B, C,. D 40x). E, F, G, H, brain frontal section of insects treated with 1.3 μg of PCBs. A general increase of NADPHd staining is observable in comparison with the control animals. The antennal lobes (al) are very intensely labelled in both the glomeruli (asterisk) and the neuropil (ne). In both the antennal lobes and the retina strongly immunoreactive and tortuous degenerative fibers (thin arrows) are present. An increase in the number of labelled glial cells (arrow heads) is evident near the calices (c) of the mushroom bodies (mb) (E, 10x; F, G, H 40x).

matic activities (Anderson, 1978; Fries and Lee, 1984; Saghir and Hansen, 1994).

Our experiments showed that PCBs induce heavy damage to many organs; in particular, we evidenced mitotic and meiotic alteration in testes, congruently with what has been observed in Musca domestica (Youssef *et al.*, 1974). Male reproductive cell pathologies were evidenced at concentrations similar to those present in animals that live in PCB-polluted environments (Niimi, 1996).

The marked PAS-positivity evidenced in testicular theca, Malpighian tubules and fat bodies, before and after diastase digestion, suggests the presence of glucide-bound proteins or lipids such as glycoproteins, glycolipids or sphingolipids.

The presence of the extensive mycotic infections in the hindgut correlated to its degeneration and to wide gaps between the epithelial cells shows how this segment of the intestine is highly sensitive to PCB action; the vast fungal formation observed in many specimens could be related to the decrease of the immunological response.

NADPH diaphorase activity, evidenced in the nervous system of treated insects, might be due to the Ca^{++}/calmodulin dependent NO-synthases (NOS). NOS produces nitric oxide (NO) which acts on cGMP in target cells and plays an essential role in many neurophysiological aspects such as feeding and behaviour, visual and olfactory stimuli coordination, learning and memory, cellular proliferation and differentiation during development (Garthwaite and Boulton, 1995). Depending on the concentration in the cells, NO can also act as a signal molecule or as a cytotoxic factor. It acts on free radicals, inducing lipid peroxidation, with consequent cell membrane damage (Griffiths *et al.*, 1998; Moncada *et al.*, 1991). This peculiar role of the molecule is evidenced in the treated specimens which, in fact, show a strong increase in NADPH-diaphorase reactivity and, likely, an increase in NO towards cytotoxic concentrations. Degenerative features, detectable in the glomeruli and in the neuropil of the antennal lobes, are proof of consequent cytological damage. The NO modification from signal molecule to cytotoxic agent goes along with the effects of pollutants and pesticides on neurotransmission (Shankland, 1979; Eldefrawi, 1985; Osborne, 1985; Hosie *et al.*, 1997; Deglise *et al.*, 2002). In fact, cellular damage due to a possible alteration in the intracellular Ca^{++} level caused by the interaction of PCBs with NMDA receptors, i.e. targets of NO, may occur (Ankarcrona *et al.*, 1995; Reynolds and Hastings, 1995; Lipton and Nicotera, 1998).

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