

REGENERATIVE RESPONSE AND ENDOCRINE DISRUPTERS IN CRINOID ECHINODERMS: ARM REGENERATION IN *ANTEDON MEDITERRANEA* AFTER EXPERIMENTAL EXPOSURE TO POLYCHLORINATED BIPHENYLS

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Summary

Regenerative phenomena, which have the advantage of reproducing developmental processes in the adult organism, are very sensitive to environmental stress and represent stages that can be monitored for damage at the whole-organism, cellular and molecular levels. Some persistent and ubiquitous pollutants, which can affect the natural environment because of their bioaccumulation in organisms, exert their effects by acting as 'endocrine disrupters'. In this respect, they can cause dysfunction in steroid hormone production/metabolism and activity by their dramatic effects on gene expression, reproductive competence and growth. The aim of our present research was to assess the impact of such compounds on adult echinoderm reproductive physiology with particular reference to regeneration potential. It is known that vertebrate-type steroids are synthesized by echinoderms and play a role in the control of growth and reproduction. Our experimental model is the crinoid *Antedon*

mediterranea, selected on the basis of its previously explored regenerative capabilities at the level of the arms. The regeneration response, analyzed at the tissue and cellular levels using both light and electron microscopy and immunocytochemistry, was employed to monitor the effects of exposure to persistent endocrine disrupter micropollutants such as polychlorinated biphenyls (PCBs) by means of laboratory tests performed under controlled conditions in terms of environmental variables and contamination levels. Our results indicate that exposure to endocrine disrupter compounds such as PCBs can induce anomalies in regeneration times, morphology and developmental mechanisms that can be interpreted in the light of significant dysfunctions in the endocrine mechanisms controlling regenerative development.

Key words: echinoderm, *Antedon mediterranea*, regeneration, morphogenesis, differentiation, polychlorinated biphenyls.

Introduction

Few species survive in environments that contain large quantities of harmful substances, but the long-term effects of low chronic contamination are no less dangerous and are certainly more insidious. Despite the widespread phenomenon of marine pollution, little is known about the effects of the low-level contamination now observed almost everywhere in the sea. The presence of harmful substances at low chronic levels may significantly affect the physiology of sensitive marine organisms, which are exposed directly to many pollutants dissolved or suspended in sea water or bound to the sediment. Exposure is generally greatest for benthic species, which are in contact with highly contaminated sediments. It is from these sediments that many pollutants are released and transferred to the aquatic compartment. Primary uptake across external epithelia (respiratory surfaces, skin, etc.) and secondary uptake from food represent important routes of entry for many dissolved aquatic pollutants.

Echinoderms are an important phylum of exclusively marine animals that offer a wide range of models for studying reproductive and developmental processes. Since echinoderms are benthic animals, they are particularly susceptible to the presence of micropollutants stored in marine sediments. Many echinoderms, besides the normal processes of sexual reproduction, frequently undergo asexual reproduction (fission) or lose parts of their bodies by defensive autotomy; these phenomena have been extensively described in the literature (Hyman, 1955; Mladenov and Burke, 1994; for a review, see Emson and Wilkie, 1980). The resultant damage/amputation is repaired promptly because of their spectacular capacity for regeneration (Thorndyke et al., 1999). These regenerative processes, which have the unique advantage of reproducing developmental processes in the adult organism, are characterized by enhanced cell proliferation, morphogenesis, differentiation and tissue renewal. For this

reason, they are amongst the biological phenomena most sensitive to environmental stress and represent stages that can be directly and conveniently monitored for cellular/tissue damage.

Some persistent and ubiquitous pollutants that can affect the natural environment because of their bioaccumulation in organisms exert their effects as 'endocrine disrupters' (Colborn et al., 1993; Fairley et al., 1996; Gray et al., 1996). They interfere synergistically or antagonistically with steroid hormone activity/metabolism and can interact with members of the nuclear receptor family, inducing dramatic effects on gene expression, reproductive competence and growth (Cooper and Kavlock, 1997; Gillesby and Zacharewski, 1998; Golub et al., 1991; Gould et al., 1997). In addition, many of these compounds that mimic natural hormone activity are only slowly metabolized and, consequently, have persistent, long-term effects in living organisms. In spite of the significant number of standardized laboratory tests with invertebrates, there is still a great demand for biomarkers specific for endocrine disrupters and for a comprehensive understanding of their potential effects.

Polychlorinated biphenyls (PCBs) are typical compounds with endocrine disrupter activity (den Besten et al., 1989; Gould et al., 1997; Voogt et al., 1990). The widespread industrial use of PCBs coupled with improper disposal has led to significant global environmental contamination (Galassi et al., 1997; Lake et al., 1995; Wania and McKay, 1993).

Although acute and chronic toxicity tests with benthic freshwater organisms are being developed for these compounds, the list of representative organisms for the marine environment is still incomplete. Echinoderms are prime candidates for this role because of their ubiquitous distribution, tractability and sensitivity. In particular, their developmental and regenerative processes represent valuable experimental models for monitoring the effects of persistent endocrine disrupter micropollutants, such as PCBs, on the developmental physiology of marine animals (Schweitzer et al., 1997) and an ideal tool to assess the growth-disrupting activity of these compounds at the tissue and cellular levels.

There is considerable published evidence suggesting that vertebrate-type steroids can be synthesized by echinoderms (Schoenmakers, 1979, 1980; Schoenmakers and Voogt, 1980; Shubina et al., 1997; Voogt et al., 1990, 1991) and that reproduction, growth and development are regulated and mediated by a combination of neuroendocrine mechanisms and local growth regulators closely analogous to those seen in the normal developmental processes of vertebrates (Anderson et al., 1994; den Besten et al., 1989). In addition, in some echinoderm classes, there is limited information on the disruptive effects of contaminants on steroid metabolism and steroid levels (Mercier et al., 1994; Meyer and Bakke, 1977), perhaps involving effects on the cytochrome P450 monooxygenase system, which is directly involved in the metabolism of steroids and lipophilic xenobiotics in both vertebrates and invertebrates (den Besten, 1998).

In the present investigation, we demonstrate that the

regenerative response of echinoderms is a valuable test for the disrupting activity of persistent micropollutants such as PCBs and contributes to our understanding of the mechanisms of action of endocrine disrupter compounds on the physiological processes of growth, differentiation and repair/regeneration at the cellular and molecular levels. Our approach employed a tractable animal model, the feather star *Antedon mediterranea*, a crinoid echinoderm representative of the marine benthic fauna and a typical microfilter-feeding animal on which persistent sediment-bound micropollutants have an immediate impact. This species is particularly amenable to the experimental study of regeneration, since it can undergo arm autotomy followed by prompt and complete regeneration of the lost arm (Clark, 1921; Reichensperger, 1912). These phenomena can be reproduced experimentally in the laboratory.

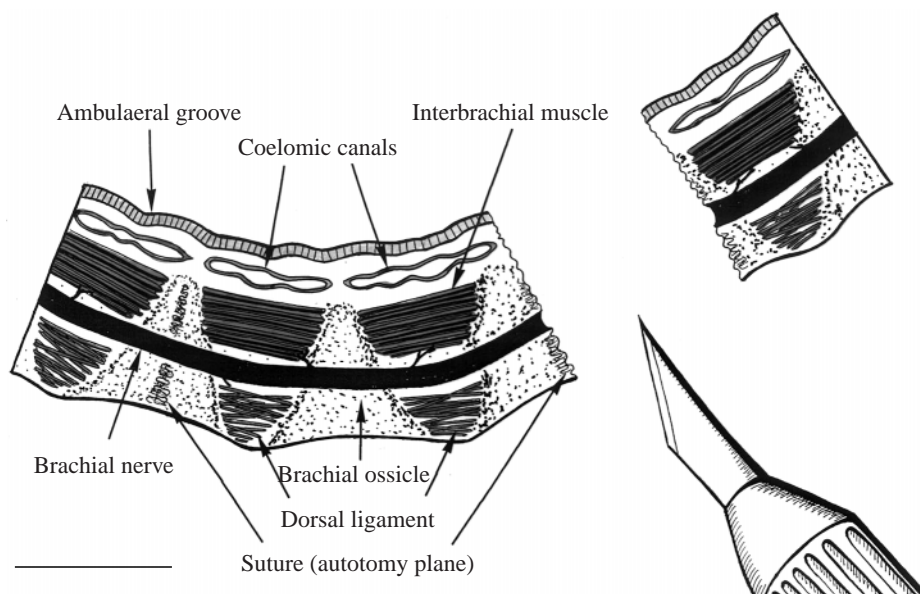
Arm regeneration in *A. mediterranea* (Candia Carnevali et al., 1993, 1995, 1997, 1998) is an epimorphic blastemal process, in which new tissues arise from the proliferation of migratory undifferentiated cells (amoebocytes and coelomocytes), and is a typical nerve-dependent regeneration, in which the nervous system acts as a primary source of regulatory factors (Candia Carnevali et al., 1996, 2000). The present account focuses on the impact of PCB exposure on crinoid arm regeneration. The experiments were carried out under controlled conditions in terms of environmental variables and contamination levels, comparable with those of moderately polluted Mediterranean coastal zones (Geyer et al., 1994). Our approach utilizes an integrated chemical and biological analysis that compares the results of the exposure tests with normal regenerating samples in control conditions, taking into account the following points: (i) bioaccumulation; (ii) effects at the whole-organism level; (iii) effects at the tissue and cellular level; and (iv) possible sites of action, particularly in terms of steroid dysfunction. In particular, a detailed histological examination at both the light and transmission electron microscope levels appears to be a rapid and sensitive method for detecting adverse effects of exposure.

Materials and methods

Exposure experiments

Specimens of *Antedon mediterranea* (Lam.), collected from the Gulf of Taranto, Italy, were maintained in aquaria in 50 l of artificial sea water at 14 °C on a diet of *Chlorella* sp. and *Tetraselmis* sp. The exposure tests to PCBs were performed in static conditions for a period of 2 weeks by employing a commercial mixture of Aroclor 1260 (Dr Ehrenstorfer G.M.D.H., Augsburg, Germany). Groups of 20 specimens were employed for each set of experiments. The experiments were repeated three times over the year. Control experiments were carried out on 20 animals in aquaria free of contaminants in parallel with exposure experiments. In both exposed and control animals, experimental regeneration was induced in three arms per specimen by amputation at an autotomy plane (Fig. 1). The regenerating samples were collected 1, 3, 7 and 14 days post-amputation. The exposure concentration was

Fig. 1. Schematic reconstruction of a sagittal section showing the detailed anatomy of the arm, which consists of a series of brachial ossicles connected by muscles and ligaments. The main continuous structures along the arm are the brachial nerve, the complex system of multiple coelomic canals and the ambulacral epithelium. Scale bar, 0.5 mm.



controlled at fixed intervals during the 2 weeks of the experiments. The PCB congener concentration tends to decrease with the exposure time. Its weighted mean was 14 ng l^{-1} as total PCBs.

Chemical analysis

The PCB congener concentrations in exposure water samples were determined after *n*-hexane extraction (10:1, v:v). The PCB bioconcentration was also analyzed in the whole animal at each stage. The lipid content was determined by mass at each stage on pooled tissues from three whole animals after Soxhlet extraction with *n*-hexane and solvent evaporation. Lipid extracts were then dissolved in 1 ml of *n*-hexane and reacted with concentrated H_2SO_4 . The hexane phase was purified on a Florisil column (4 cm \times 0.7 cm) and analysed by high-resolution gas chromatography with electron capture detector (Provini and Galassi, 1999).

Biological analysis

The biological analysis employed both standard methods for morphological analysis by stereomicroscope, light microscope and transmission electron microscope, as described in detail by Candia Carnevali et al. (1993), and specific immunocytochemical methods including protocols for monitoring cell proliferation (5-bromodeoxyuridine, BrdU, method; Candia Carnevali et al., 1995, 1997, 1998).

Results and discussion

Bioconcentration

The pattern of individual PCB uptake in *A. mediterranea* during the different regenerative stages over 14 days is shown in Fig. 2A. PCB congeners are numbered according to the system of Ballschmiter and Zell (1980). The sharp increase over 14 days in concentration of all PCB congeners (normalised to total lipid content) observed in the animal

tissues is typical of the initial phases of uptake in bioconcentration experiments (Connell, 1990). Although, at present, the equilibration times for PCBs in echinoderms are unknown, in other marine animals, such as molluscs and fish, the expected equilibrium times for the less-hydrophobic PCBs (such as congeners 101 and 110; Hawker and Connell, 1988), should be 1 month and 1 year, respectively (Connell, 1990). Even longer equilibration times are expected for the other more-hydrophobic congeners (Connell, 1990). The PCB profile in *A. mediterranea* differed significantly from that of the commercial mixture of Aroclor 1260 used to prepare the exposure water (Fig. 2B). An enrichment of less-chlorinated PCBs (again, such as congeners 101 and 110; Hawker and Connell, 1988) was observed in the animal tissues both because these congeners are more soluble in water and because they bioconcentrate more quickly (Connell, 1990). The 14-day total PCB concentration was 2257 ng g^{-1} lipid, not very different from those measured in other filter-feeding invertebrates collected along Mediterranean coasts (Geyer et al., 1994; Galassi et al., 1993; Picer and Picer, 1991).

Gross morphology, microscopic anatomy and immunocytochemistry

During the repair phase, 1 day post-amputation, and during the early regenerative phase (2–3 days post-amputation), the PCB-exposed samples showed no significant anomalies in terms of general morphology and external anatomy compared with the controls (Candia Carnevali et al., 1999). There was a slight delay in the processes of cicatrization/re-epithelialization of the amputation surface and in the overall growth of the regenerating blastema. In contrast, at the more advanced regenerative stages (7 days post-amputation), abnormal growth of the regenerate was clearly evident in all the exposed samples (Fig. 3A,B). This abnormal growth involved the overall size of the regenerating arm, which appeared to be much more developed, and the differentiation of its typical anatomical

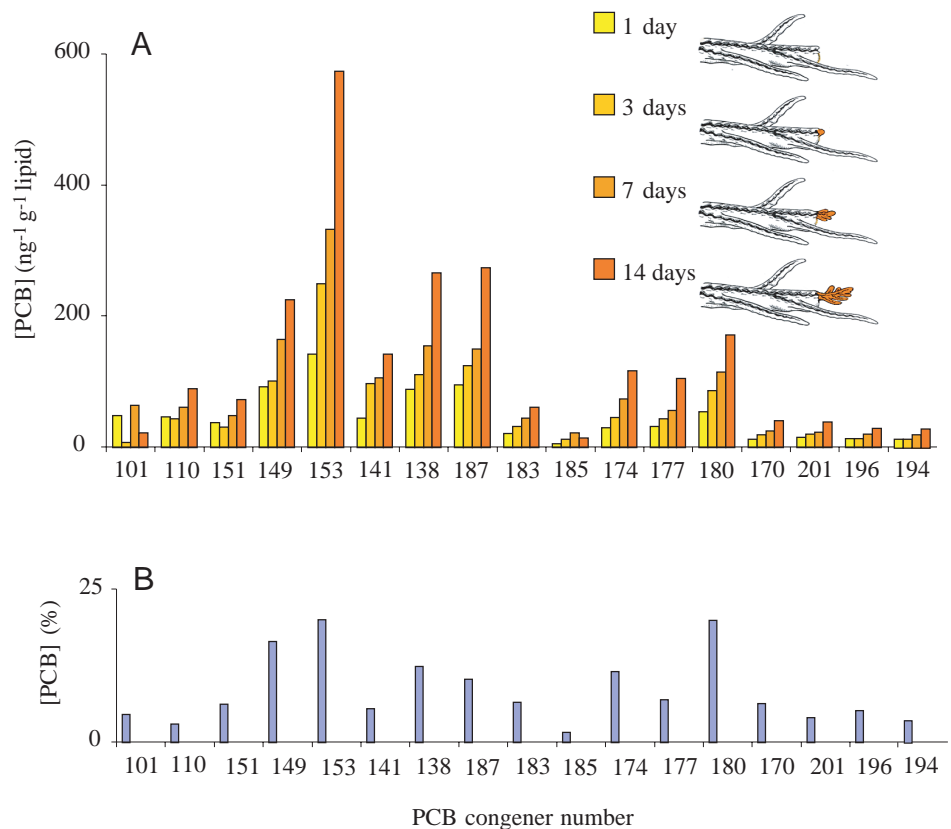


Fig. 2. Chemical analysis for polychlorinated biphenyls (PCBs). (A) Concentrations of PCB congeners in *Antedon mediterranea* (whole organism) at four different regenerative stages of the overall 14 day post-amputation period. (B) PCB composition of the commercial mixture of Aroclor 1260 used to prepare the exposure medium. PCB congeners are numbered according to the system adopted by Ballschmiter and Zell (1980).

structures, both external (lateral pinnules) and internal (brachial ossicles). These anomalies became progressively more evident at 14 days post-amputation, emphasizing an impressive increase in terms of overall growth and development of all the PCB-treated samples in comparison with the controls.

These effects at the level of general morphology could be correlated with the appearance of unusual features at the level of microscopic anatomy, which involved the histological and cellular patterns of both the stump and the regenerating arm. At the tissue level, in comparison with the control samples, the

following features were particularly significant. (i) There was hypertrophic development and marked swelling of the coelomic canals in both the regenerate and the stump (Fig. 4A,B). (ii) There was massive and prolonged cellular proliferation/migration involving the migratory cells responsible for both repair and regeneration (coelomocytes, amoebocytes, phagocytes, granule cells; Candia Carnevali et al., 1993, 1995, 1997). As indicated by BrdU incorporation experiments, these phenomena were localized not only at the level of the usual sites of cell recruitment, i.e. the coelomic epithelium and the brachial nerve of both the regenerate and the stump, and the apical

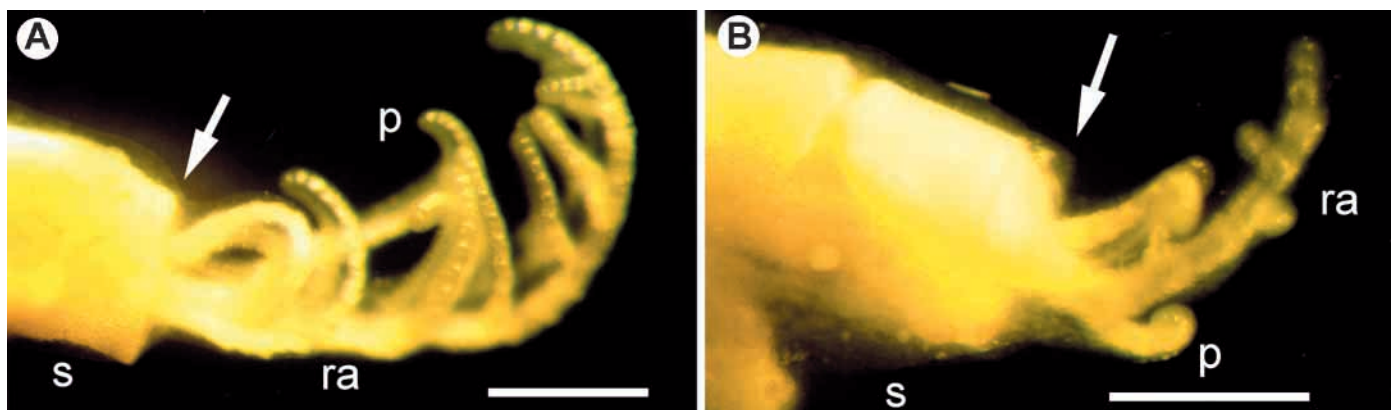


Fig. 3. Comprehensive stereomicroscopic views of regenerating arms of *Antedon mediterranea* at 14 days post-amputation. (A) A polychlorinated biphenyl (PCB)-exposed sample showing the abnormal growth of the regenerate and the advanced development of its anatomical features. p, pinnules; s, stump; ra, regenerating arm; arrow, amputation plane. (B) Control sample. Scale bars, 0.5 mm.

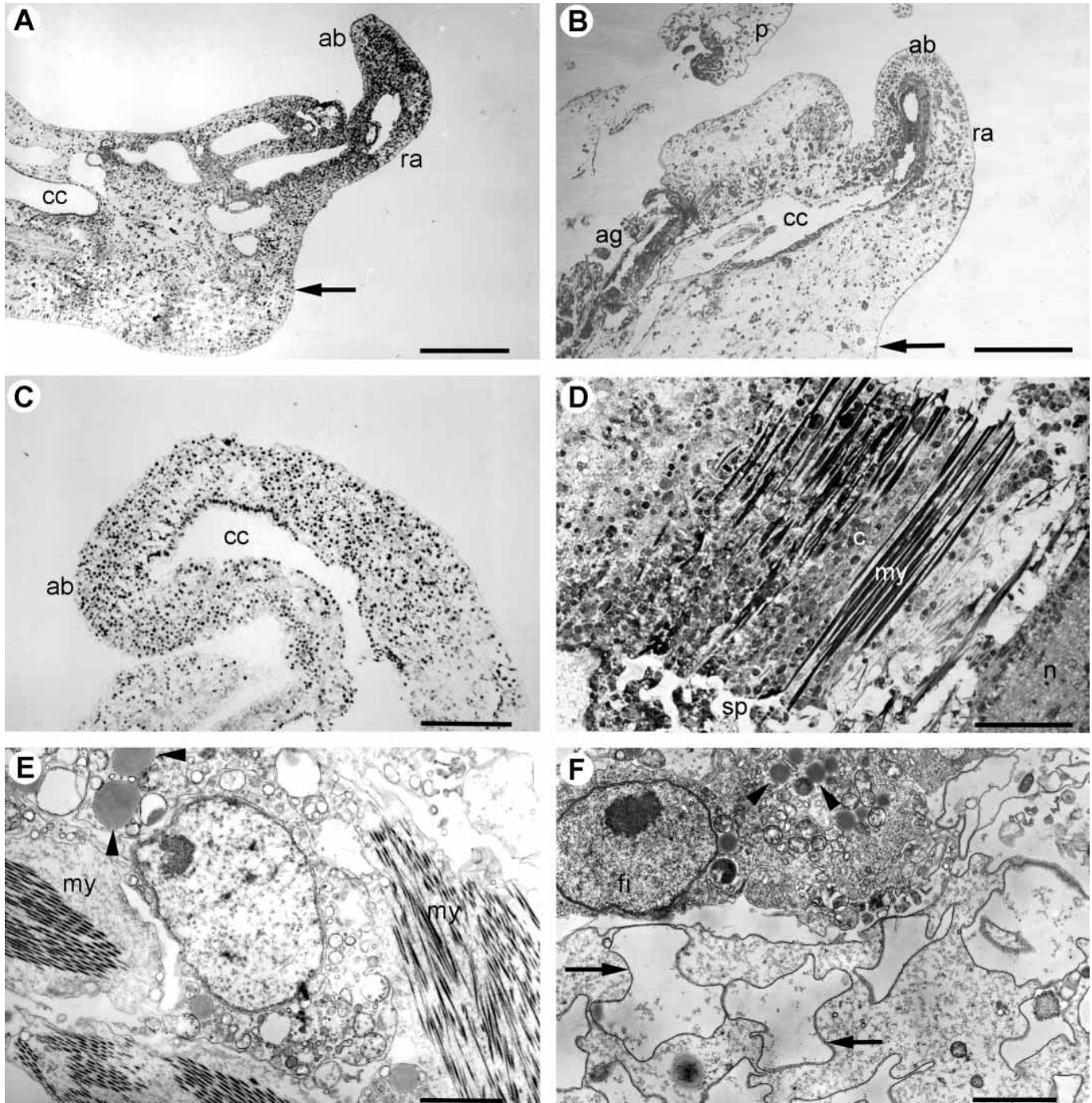


Fig. 4. Histological and cellular aspects of the effects of polychlorinated biphenyls (PCBs). (A) Light micrograph of a PCB-exposed regenerating sample at 7 days post-amputation. A sagittal section showing the overall abnormal growth of the regenerate and marked hypertrophic development of the coelomic canals. ab, apical blastema; cc, coelomic canal; ra, regenerating arm; arrow, amputation plane. Scale bar, 200 μ m. (B) Light micrograph of a control sample at 7 days post-amputation. ag, ambulacral groove; p, pinnule. Scale bar, 160 μ m. (C) Light micrograph of a sagittal section of a PCB-exposed regenerating sample at 7 days post-amputation. Immunocytochemistry for bromodeoxyuridine. The blastemal cells and the coelomic epithelium were heavily labelled. Scale bar, 100 μ m. (D) Light micrograph of a sagittal section of a PCB-exposed regenerating sample at 7 days post-amputation. Detail of the muscle bundle at the stump level showing tissue rearrangement/dedifferentiation. c, coelomocytes; my, myocytes; n, brachial nerve; sp, skeletal spicule. Scale bar, 60 μ m. (E) Transmission electron micrograph of a PCB-exposed sample at 7 days post-amputation. Detail of the muscle at the stump level. There is evidence of dedifferentiation processes involving individual myocytes (my). Many vacuoles and lipid granules (arrowheads) are also present. Scale bar, 100 μ m. (F) Transmission electron micrograph of a PCB-exposed sample at 7 days post-amputation. Detail of the connective tissue at the stump level showing extensive tissue rearrangement/degeneration, which gives rise to lace-like figures (arrows). This cellular degeneration cannot be attributed to poor preservation of the tissue, as indicated by the good quality of cell preservation/fixation of the adjacent fibroblast (fi); arrowheads, lipid granules. Scale bar, 30 μ m.

blastema of the regenerating arm (Fig. 4C), but unexpectedly also at the level of some differentiated tissues of the stump, particularly the muscles. (iii) There was extensive rearrangement and/or dedifferentiation, specifically involving some tissues of the stump, namely the muscles, the endoskeleton and the connective tissue (Fig. 4D,E,F). At the level of the muscle bundles of the stump (Fig. 4D), in particular, the muscle fibres appeared to lose their packed and compact structure and were extensively replaced by other elements such as coelomocytes and phagocytes.

At the cellular level, these anomalies were confirmed and associated with an atypical cytological pattern. This involved mainly the cell components of the rearranging tissues of the stump. In the muscles, in particular, the individual myocytes had lost their contractile characteristics (Fig. 4D,E) and seemed to dedifferentiate progressively, acquiring the undifferentiated features of migrating coelomocytes actively involved in cell division, as shown by BrdU labelling. This phenomenon, never observed in the controls, closely corresponded to that previously described in other extreme conditions of regeneration, i.e. in the case of arm explants (Candia Carnevali et al., 1998). Thus, in PCB-exposed samples and in other conditions of stress, the muscles seem to provide a significant morphallactic contribution to regeneration in terms of reserve cells to be employed for tissue renewal. This is typical of morphallactic regeneration: the involvement of cells derived from existing tissues by dedifferentiation/transdifferentiation and migration (Thorndyke et al., 1999). It is important to emphasize that this phenomenon was never observed in control conditions, where regeneration is typically accomplished by undifferentiated stem cells (Candia Carnevali et al., 1995, 1997).

At the level of the connective and the endoskeletal tissue (Fig. 4F), in contrast, tissue rearrangement involved mostly extensive degeneration phenomena, leading to an appreciable vacuolization/vesiculation of both the extracellular matrix and the cells (fibroblasts or scleroblasts), and was characterized by lace-like remnants of cell processes and membranes. The abundant and unusual presence of phagocytes and granule cells (i.e. elements typically employed in repair processes), even at the advanced regenerative stages at 7–14 days, indicated that the connective tissues seemed to be employed as a secondary indirect source of reserve materials for new synthesis rather than for producing undifferentiated cells for regeneration. These extensive and striking phenomena of tissue rearrangement and dedifferentiation at the level of the stump will be explored appropriately in a more detail in a further development of this research (M. D. Candia Carnevali, F. Bonasoro, P. Ferreri and S. Galassi, in preparation).

In contrast, at the level of the regenerating arm, no significant histological anomalies were evident and, in particular, differentiation of tissues and cell lines (i.e. specialised epithelia and associated nerve plexuses, musculo-skeletal components, central and lateral nerves) progressed normally with no significant variation compared with control conditions. However, some unusual features were evident in

the ultrastructural pattern of many cell types, including the blastemal cells. In particular, the marked development of endoplasmic reticulum (both rough and smooth), the extensive presence of Golgi complexes, the unusually abundant lipid granules and the empty vacuoles are all features that could be attributed to the significant activation of cell mechanisms related to steroid synthesis/metabolism (Krstic, 1979; Motta, 1984) and/or to detoxification processes (Schoenmakers, 1980; den Besten, 1989).

It is relevant that recent results have been obtained with regard to the expression of biomarkers that are recognized indicators of possible protective biochemical responses, in terms of possible mechanisms controlling the levels of free pollutants in the organism or concerning the repair of damage caused by pollutants. The data obtained so far show that, in PCB-exposed samples, there was an appreciable increase in the expression patterns of (i) 'stress' proteins such as ubiquitin (Patrino et al., 2001), which can be induced in response to the protein damage and aggregation resulting from a variety of environmental stressors and (ii) specific enzymes such as the microsomal cytochrome P450 mono-oxygenase system (Candia Carnevali et al., 2000), which are the main enzymes responsible for biotransformation and metabolism of the majority of lipophilic xenobiotics. Thus, these preliminary results clearly reflect prior toxicant-induced molecular and biochemical aspects of cell physiological alterations.

In parallel with the qualitative approach, a quantitative analysis of the morphological changes induced by PCB exposure was carried out on the data collected from each set

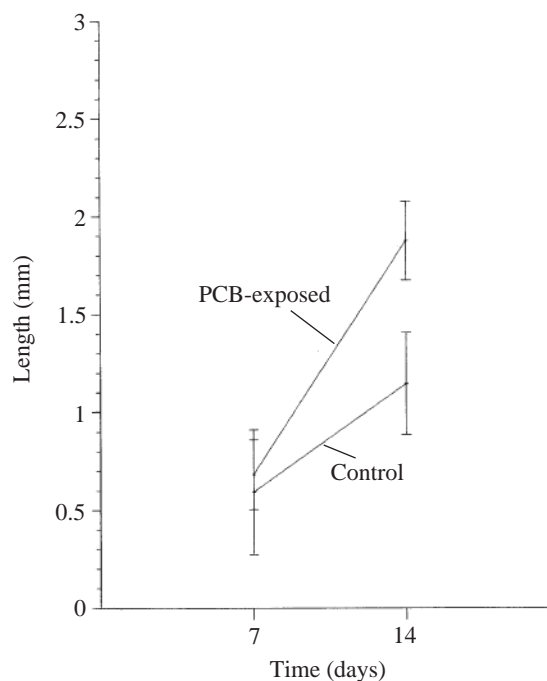


Fig. 5. Mean lengths of the regenerating arms recorded at 7 days and 14 days post-amputation for PCB-exposed samples and controls. The differences in length recorded were statistically significant ($P < 0.05$) at 14 days post-amputation. Values are means \pm S.E.M. ($N=160$).

Table 1. Statistical analysis of the measured lengths (mm) of the regenerating arms of *Antedon mediterranea*

Source of variation	d.f.	Sum of squares	Mean square	F-ratio	P
Treatment	1	1.1404328	1.1404328	11.685	0.0019
Time	1	1.1665468	1.1665468	52.935	0.00001
Treatment \times time	1	0.6926954	0.6926954	7.097	0.0125
Residual	29	2.8304523	0.0976018		
Total (corrected)	32	12.195933			

Treatments were PCB exposure *versus* control; times were 7 *versus* 14 days post-amputation.

The data refer to the third set of experiments.

F-ratios are based on the residual mean square error.

of experiments, with particular reference to the measured lengths of the regenerating arms in PCB-exposed samples and in the controls (Fig. 5). Measures related to regenerates 7 days and 14 days post-amputation were considered separately for each set of experiments. Analysis of variance (Sokal and Rohlf, 1995) was performed on two factors (ANOVA): treatment (PCB-exposed and control) and regeneration time (7 days and 14 days). This analysis (Table 1) showed clearly that both these factors were highly significant ($P < 0.002$) and that their interaction was also significant ($P = 0.0125$). These results are emphasized in Fig. 5, which shows that the mean regenerate lengths in the PCB-exposed samples and in controls were significantly different ($P < 0.05$) at 14 days post-amputation.

In conclusion, our present results provide clear evidence that prolonged exposure to low concentrations of PCBs, typical of moderately polluted coastal zones, results in marked bioaccumulation in benthic macroinvertebrates, such as crinoid echinoderms, and affects their physiology by interacting with mechanisms regulating growth and cell proliferation/turnover. Moreover, PCB exposure appears to induce specific modifications and anomalies in regenerative developmental processes that, in terms of general growth and tissue/cellular aspects, are compatible with a pattern of pseudo-endocrine activities and steroid dysfunction. The regenerative response of crinoid echinoderms provides a sensitive test for contamination by endocrine disrupter pollutants and highlights novel aspects and mechanisms of toxicity of such compounds. In particular, exposure to these types of toxic chemicals can result in (i) significant variations in the timing and mechanisms of arm regeneration, (ii) abnormal growth and (iii) anomalous developmental processes at the tissue and cellular levels. The extensive cell proliferation and the tissue rearrangement phenomena found in the PCB-treated samples confirms that exposure to endocrine disrupters significantly affects the regulation of growth by directly interacting with the cascade of cellular processes involved in the cell cycle and tissue turnover. In addition, we suggest that exposure to PCBs may induce detoxification processes and related nuclear receptor activities similar to those known in other invertebrates. The development of specific and direct molecular monitoring methods for exploring endocrine-disrupter-induced modulations of nuclear receptor activities in echinoderms will be a useful tool to throw light on these points.

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