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Review

Endocrine disruption in crustaceans due to pollutants: A review ☆

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Abstract

The main endocrine-regulated processes of crustaceans have been reviewed in relation to the effects of endocrine-disrupting compounds (EDCs). Molting has been shown to be inhibited by several organic pollutants, such as xenoestrogens and related compounds, as well as by some pesticides. Most of these disrupters are thought to interfere with ecdysone at target tissues, although only for a few has this action been demonstrated in vitro. The heavy metal cadmium appears to inhibit some ecdysone secretion. Juvenoid compounds have also been shown to inhibit molting, likely by interfering with the stimulatory effect of methyl farnesoate. A molt-promoting effect of emamectin benzoate, a pesticide, has also been reported. As for reproduction, a variety of organic compounds, including xenoestrogens, juvenoids and ecdysteroids, has produced abnormal development of male and female secondary sexual characters, as well as alteration of the sex ratio. Cadmium and copper have been shown to interfere with hormones that stimulate reproduction, such as methyl farnesoate, as well as with secretion of the gonad inhibiting hormone, therefore affecting, for example, ovarian growth. Several heavy metals were able to produce hyperglycemia in crustaceans during short times of exposure; while a hypoglycemic response was noted after longer exposures, due to inhibition of secretion of the crustacean hyperglycemic hormone. The ecological relevance of EDCs on crustaceans is discussed, mainly in relation to the identification of useful biomarkers and sentinel species. New experimental approaches are also proposed.

Keywords: Endocrine disruption; EDCs; Crustaceans; Growth; Molting; Reproduction; Glycemia

Contents

1. Introduction

The volume of published literature about endocrine disruption in wild species has increased significantly during the last decade. Many cases have been reported, both in vertebrate and invertebrate animals (Kendall et al., 1998; DeFur et al., 1999; Oberdörster and Cheek, 2000; Oetken and Bachmann, 2004).

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However, studies on the specific mechanisms by which the endocrine system can be disrupted are scarce, and this is a critical point to be developed for fully understanding the risk of known endocrine-disrupting compounds (EDCs), as well as for predicting the potential deleterious effects of pollutants whose disrupter effects on endocrine systems are not still evident.

In fact, endocrine disruption can take place at different physiological levels: A) altering (inhibiting or stimulating) the secretion of hormones. This possible effect is related to mechanisms that control both the release of hormones from endocrine cells and synthesis of these hormones: B) interfering with hormone-receptor interaction. In this sense, an EDC can act as an agonist or antagonist by directly binding to a hormone receptor. Indirectly, though, an EDC could interfere by several mechanisms at any step of the transductional pathway of a hormone, therefore altering its final effect: C) modifying the metabolism of circulating hormones, that is, by increasing or decreasing their excretion rate and/or biotransformation in the liver, hepatopancreas or other organs.

A wide range of pollutants have been reported to be EDCs for invertebrate species, especially organics such as alkylphenols (that can act as estrogenic compounds), polychlorinated biphenyls (PCBs), chlorinated pesticides, herbicides and petroleum hydrocarbons. Natural steroids such as estrogens when present in sewage discharge can be also affect vertebrate aquatic species (Oberdörster and Cheek, 2000; Depledge and Billinghurst, 1999). Heavy metals have not been studied as frequently, but recent reports (Bondgaard and Bjerregaard, 2005; Rodríguez Moreno et al., 2003; Medesani et al., 2004a) stress the risk that this kind of pollutant is indeed an EDC. The organometallic compound tributyltin (TBT) has classically been reported to be a strong EDC, producing masculinization of gastropod females probably by interfering with more than one mechanism, i.e., by inhibiting P450-dependent aromatase that converts endogenous testosterone to estradiol, as well as by inhibiting testosterone excretion, therefore giving rise to a phenotype known as imposex (Matthiessen and Gibbs, 1998).

Invertebrates constitute 95% of all the known animal species in nature; despite this, most of the in vitro bioassays used for identifying EDCs (xenoestrogens, for instance) have been developed with vertebrate (particularly mammalian) cell lines or receptors (Depledge and Billinghurst, 1999). More recently, an in vitro assay using the ecdysteroid receptor of the fruit fly *Drosophila melanogaster* has been developed to test possible interactions of pollutants on that receptor (Hutchinson, 2002; Pounds et al., 2002). Because of the relatively close phylogenetic affinity of insects and crustaceans, this assay is undoubtedly a useful tool for evaluating the potential effects of xenobiotic on molting and other processes controlled by ecdysteroids in crustaceans. Certainly, integration of in vivo and in vitro assays is essential for obtaining results with a high ecotoxicological relevance.

Crustaceans are one of the most ubiquitous groups of invertebrates, inhabiting all types of aquatic habitats. They have been chosen as test species for evaluating EDC effects in numerous studies (Kendall et al., 1998; DeFur et al., 1999; Oberdörster and Cheek, 2000; Depledge and Billinghurst, 1999). Hormonally-regulated functions affected by pollutants have been previously reviewed in crustaceans by Fingerman et al. (1998) and Zou and Fingerman (2003). The aim of the current review is to update the state of the art, emphasizing from a systemic point of view the critical steps and mechanisms of the endocrine system of crustaceans potentially affected by environmental pollutants. The main endocrine-regulated processes will be separately considered.

2. An overview of crustacean endocrinology

In regard to their endocrine systems, invertebrates have been relatively far less studied than vertebrates, with most of the literature published on invertebrate endocrinology referring to insects and crustaceans. The earliest evidence of crustacean hormones was provided during the 1920s, when the endocrine nature of the eyestalks became evident. The X-organ–sinus gland complex is the main neuroendocrine organ of crustaceans. The X-organ is part of the *medulla terminalis* in the eyestalk, and projects axons to the neurohemal organ, the sinus gland, that lies between the *medulla externa* and *medulla interna* in the eyestalks. In crustaceans with no differentiated eyestalks, the X-organ–sinus gland complex is located in homologous structures of the central nervous system in the head. In all cases, hormones produced by the X-organ are stored in the axonal terminals that reach and compose the sinus gland, to be secreted under the appropriate stimuli. The main hormones secreted by the sinus gland are the following: MIH (molt inhibiting hormone), GIH (gonad inhibiting hormone), MOIH (mandibular organ inhibiting hormone), CHH (crustacean hyperglycemic hormone), several color change hormones (controlling pigment migration) and NDH (neurodepressing hormone). Some of these hormones have a second endocrine gland as their target (MIH, GIH, MOIH), while the others have somatic tissues as targets. MIH, GIH, MOIH and CHH belong to a single family of peptides, actually showing some cross reactions among themselves (Keller, 1992; Webster, 1998; Chan et al., 2003).

Molting is controlled by MIH acting on the so-called Y-organs, a pair of structures homologous to the prothoracic organs of insects. Both the Y-organs and prothoracic organs are non-neural endocrine organs that produce and secrete ecdysone. In the case of crustaceans, ecdysone is peripherally transformed into the active hormone 20-hydroxyecdysone. MIH exerts an inhibitory effect on the Y-organ by reducing the intracellular level of the second messenger cAMP; the decrease of circulating MIH at the beginning of premolt triggers the secretion of ecdysone by the Y-organ (Mattson and Spaziani, 1986). In addition, new evidence indicates that the Y-organ becomes unresponsive to MIH during premolt (Chung and Webster, 2003), especially during the second half of this period (Nakatsuji and Sonobe, 2004). Hemolymphatic calcium levels also modulate ecdysone secretion by the Y-organs (Spaziani et al., 1999).

On the other hand, the juvenile hormone (JH) of crustaceans, i.e., methyl farnesoate (MF, Laufer et al., 1987) is a sesquiterpenoid compound (the unepoxidated form of JHIII of insects), secreted by the mandibular organs, whose secretion is inhibited by the eyestalk hormone MOIH. In vitro stimulation by MF of the Y-organ, resulting in ecdysone secretion, has been reported (Tamone and Chang, 1993). Rodríguez et al. (2002a) have reported that molting in crayfish is stimulated by JHIII administration, JHIII perhaps having been converted to MF. In fact, several functions

involving both molting and reproduction have been proposed for MF (Homola and Chang, 1997). Fig. 1 illustrates the roles of these hormones in molting control.

Endocrine control of reproduction is a more complex phenomenon. In females, the eyestalk hormone GIH directly inhibits the oocytes, regulating the uptake of vitellogenin from the hemolymph during secondary vitellogenesis (Charniaux-Cotton and Payen, 1988). GIH would appear to also inhibit the secretion of one or more ovarian hormones. Evidence for a peptidic "ovarian hormone" was initially proposed for amphipods (Charniaux-Cotton and Payen, 1988). Steroids as hormone products of the ovary have been suggested by several authors (reviewed by Fingerman et al., 1993). Some studies showed circulating levels of progesterone and estradiol-like steroids that correlated with the vitellogenesis cycles of several crustaceans (Quinitio et al., 1994; Shih, 1997). Both in vivo (Rodríguez et al., 2002a; Zapata et al., 2003) and in vitro (Tsukimura and Kamemoto, 1991; Zapata et al., 2003) stimulation of ovarian growth by 17α-hydroxyprogesterone has been reported for some crustacean species. The precise role of steroid hormones in crustaceans is still unknown; however, stimulation of vitellogenin production within the ovary itself or in extra-ovarian sites has been suggested (Quackenbush, 1994; Yano, 2000; Rodríguez et al., 2002a).

In addition, neuroendocrine centers outside the eyestalks also participate in the regulation of crustacean reproduction. Among them, the brain and thoracic ganglia have been identified as sources of a neurohormone called GSH (gonad stimulating hormone). Although GSH has not yet been purified, several in vivo and in vitro studies showed the stimulating effects of the brain and thoracic ganglia on ovarian growth (reviewed by Fingerman, 1997). Some neurotransmitters have been found to either stimulate or inhibit secretion of GSH, serotonin and dopamine respectively. These and other neurotransmitters also influence the secretion of eyestalk hormones, including GIH (Fingerman, 1997). An isoform of CHH, identified by immunocytochemistry in the ventral nerve cord of some crustaceans may possible function here as the GSH (De Kleijn and Van Herp, 1998; Charmantier et al., 1997).

MF has been shown to have a role in crustacean reproduction. Administration of MF, incorporated in food, resulted in the stimulation of ovarian growth in crayfish (Laufer et al., 1998). Similarly, pellets enriched with JHIII simulated ovarian growth in crabs (Zapata et al., 2003). In vitro stimulation of ovarian growth by MF has been reported for both penaeid shrimp (Tsukimura and Kamemoto, 1991) and crayfish (Rodríguez et al., 2002b). In addition, ecdysteroids secreted by the Y-organ may also play a role in stimulating ovarian growth, especially in those crustaceans where reproduction occurs just after molting, as in penaeid shrimps. In other crustaceans, ecdysteroids may also be playing a role in reproduction, but it is restricted to the first, i.e., proliferative stage of ovarian growth (Adiyodi and Subramonian, 1983). Fig. 2 shows a model of the possible endocrine control of crustacean female reproduction.

Reproduction of crustacean males involves a special endocrine gland, the androgenic gland. This is a paired structure, one being

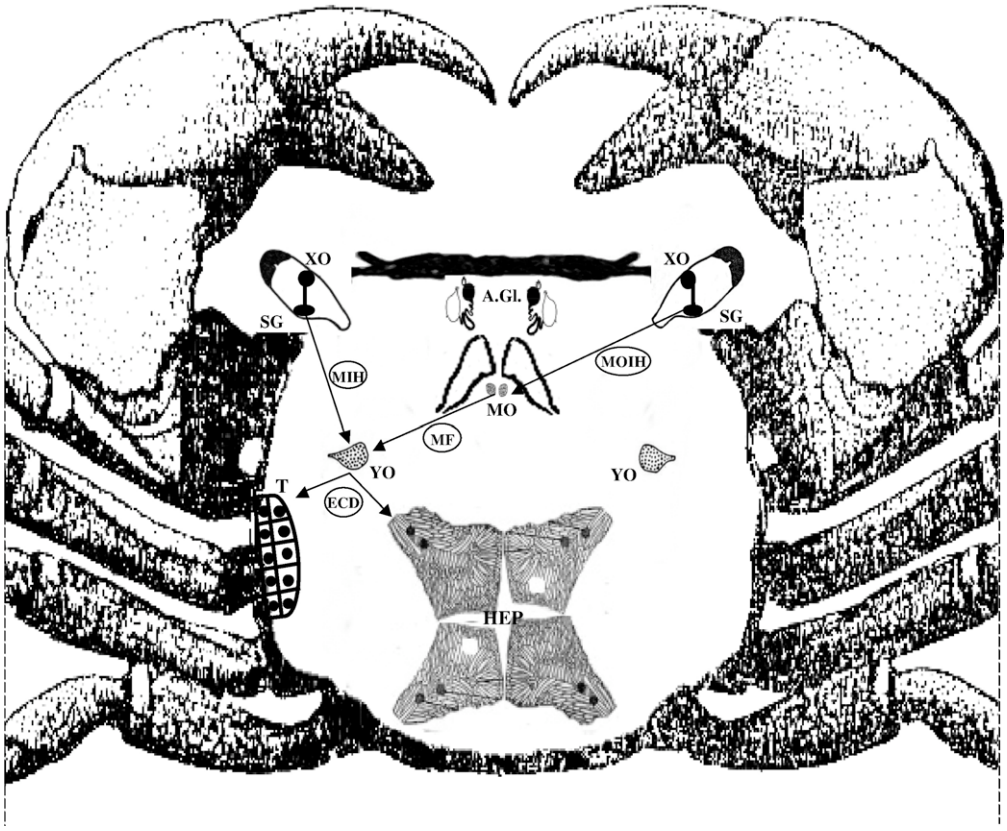

Fig. 1. Endocrine control of crustacean molting. XO: X-organ, SG: sinus gland, YO: Y-organ, MO: mandibular organ, HEP: hepatopancreas, T: integument, A.Gl.: antenal gland, MIH: molt inhibiting hormone, ECD: ecdysone, MOIH: mandibular organ inhibiting hormone, and MF: methyl farnesoate.

attached to each vas deferens. The testes are directly controlled by a hormone secreted by the androgenic gland, called the androgenic gland hormone (AGH), with GSH and GIH influencing the secretion of AGH, instead of directly acting on the testes. AGH determines the normal development of both the male reproductive system and the male secondary sexual characters (Fingerman et al., 1998). Fig. 2 illustrates this endocrine control.

Increased circulating levels of CHH cause an increment in the glucose hemolymphatic concentration, mainly by stimulating glycogen breakdown in the hepatopancreas (Santos and Keller, 1993). In addition, CHH has been shown to possibly mediate water uptake at molting, as well as to stimulate oocyte growth during the onset of vitellogenesis (Fanjul-Moles, 2006, for review). Color changes that depend on small peptides whose function is to promote the dispersion or concentration of pigments in several kinds of epithelial chromatophores have been reported; some of these peptides also regulate the migration of the distal pigment in the ommmatidia of the eyes, in response to changes in light intensity (Fingerman et al., 1998).

3. Effects of EDCs on molting and growth

The effect of estrogenic EDCs on crustacean molting has been the subject of several studies. Zou and Fingerman (1997a) found a delay in molting of neonate *Daphnia magna* evoked by the synthetic estrogen diethylstilbestrol (DES), as well as by the estrogenic pesticide endosulfan. A similar effect has been also reported for other estrogenic compounds, such as the polychlorinated biphenyl (PCB) Aroclor 1242 and diethyl phthalate (Zou and Fingerman, 1997b). Further studies by the same authors showed that all of these xenoestrogens significantly inhibited chitobiase activity in the epidermis of the fiddler crab, *Uca pugilator* (Zou and Fingerman, 1999a,b). This enzyme is necessary for the partial digestion of the chitinous cuticle during premolt, its activity being under the control of the molting hormone ecdysone. Therefore, it is quite possible that the delay in molting caused by these xenoestrogens is due to their blocking of the ecdysteroid receptor in the epidermis.

Also working with *D. magna*, Mu and LeBlanc (2002a) reported a delay of molting of early instars due to testosterone exposure. The levels of circulating ecdysone, measured by means of a radioimmunoassay, were not changed by the exposure to testosterone. However, testosterone was able to antagonize the effect of 20-hydroxyecdysone on an ecdysone-responsive cell line of *D. melanogaster*. These results strongly suggest that testosterone delays molting by blocking the ecdysteroid receptor. However, the agricultural fungicide fenarimol that has been shown to have anti-ecdysteroidal activity on *D. magna*, has a

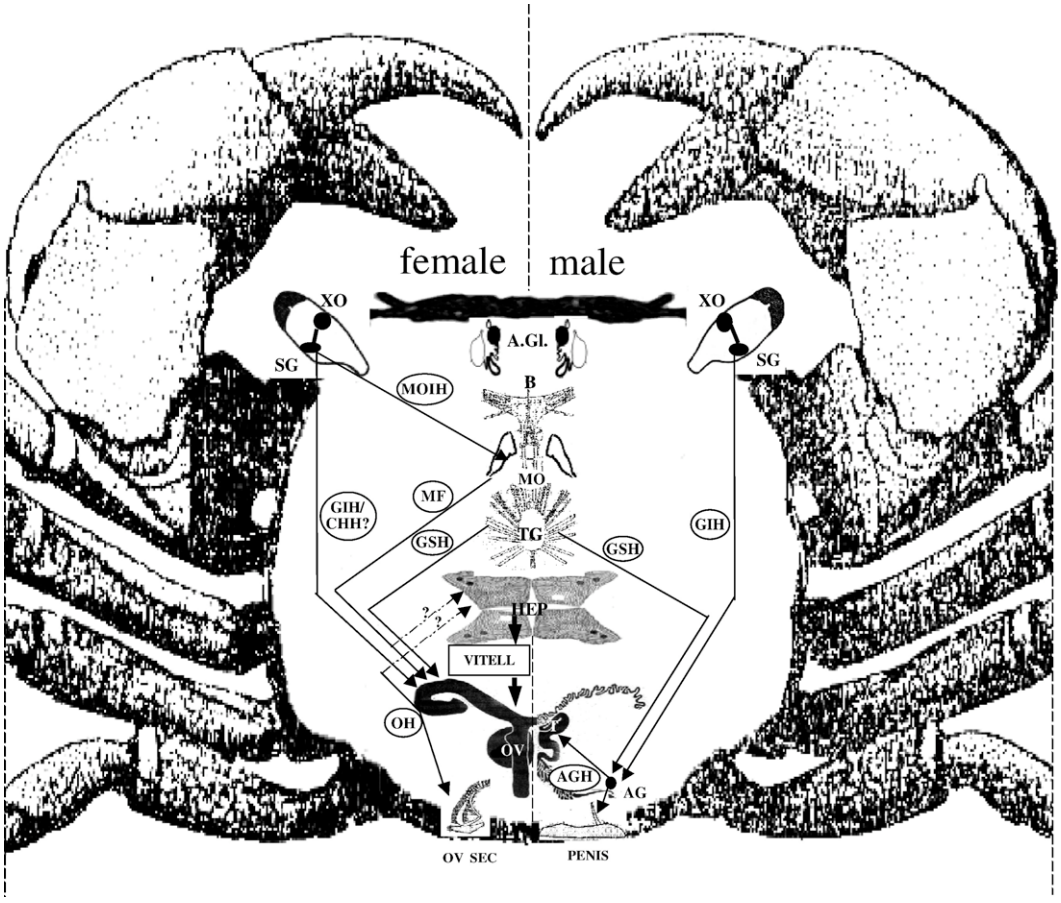

Fig. 2. Endocrine control of crustacean reproduction. XO: X-organ, SG: sinus gland, MO: mandibular organ, B: brain, TG: thoracic ganglia, HEP: hepatopancreas, OV: ovary, T: testis, AG: androgenic gland, A.Gl.: antenal gland, GIH: gonad inhibiting hormone, GSH: gonad stimulating hormone, MOIH: mandibular organ inhibiting hormone, CHH: crustacean hyperglycemic hormone, MF: methyl farnesoate, OH: ovarian hormone, AGH: androgenic gland hormone, and VITELL: vitellogenin.

different mode of action; it reduces the circulating levels of ecdysone (Mu and LeBlanc, 2002b).

Copepods are another group of crustaceans that have been used as a model for assays of EDCs. Andersen et al. (2001) reported an inhibition of naupliar development (i.e., the molting and metamorphosis from nauplii to the copepodite stage) of *Acartia tonsa* by exposure to endocrine disrupters, the xenoestrogens 17α-ethinylestradiol and p-octylphenol, the antiestrogen tamoxifen and the antiandrogen flutamide. These investigators suggested that these inhibitors act on the ecdysone receptor, thereby raising the threshold of ecdysone needed to trigger molting. However, no experimental data that support this hypothesis was provided.

In another study on copepods, Dinan et al. (2001) reported the results of a wide variety of environmental pollutants, assayed in vitro for their binding with the ecdysone receptor, using an ecdysteroid-responsive hemocyte cell line from *D. melanogaster*, as mentioned above. In this assay, cell proliferation is taken as the endpoint: agonist effect was evaluated vs. a control with no hormone added, while an antagonist effect was evidenced by inhibition as compared with a 20-hydroxyecdysone treated group. Among 80 compounds assayed only three showed a strong antagonist effect, i.e., the estrogenic compounds bisphenol A, diethylphtalate and lindane, at low concentrations. The copepod *Tisbe battagliai,* was also used to carry out in vivo assays for assessing the sublethal effects of pollutants on growth and reproduction throughout the entire 21-day life cycle of the species (Pounds et al., 2002; Hutchinson, 2002). Many of the compounds that significantly antagonized the ecdysone receptor did not produce any effect in vivo, presumably due to a restricted uptake, efficient catabolism and/or excretion. On the other hand, the synthetic estrogen diethylstilbestrol inhibited molting in vivo but showed no effect in vitro, indicating a mode of action other than interaction with the ecdysone receptor (Pounds et al., 2002).

Snyder and Mulder (2001) reported a delay in the onset of molting of larvae of the lobster *Homarus americanus* exposed to heptachlor. This delay was correlated with both reduced levels of circulating ecdysteroids and increases of some P450-dependent detoxifying enzymes known collectively as CYP45. Although it is known that 20-hydroxyecdysone itself can induce the expression of these enzymes, it is quite possible that this induction can also be produced by some EDCs.

Inhibition of molting is not always caused by pollutants. Waddy et al. (2002) observed precocious molting by female lobsters *H. americanus* in response to emamectin benzoate, a food additive used to control sea lice infestations in fishes. Since this compound causes a delay of molting of insects, the authors hypothesized that this pollutant acts by inhibiting the release of a neuropeptide that controls molting, both in insects where molting is initiated by a stimulatory neuropeptide and crustaceans where molting is controlled by the inhibitory neuropeptide eyestalk hormone MIH. The fact that emamectin benzoate is a GABAergic pesticide, and that GABA can inhibit the secretion of several eyestalk hormones (Sarojini et al., 2000) supports this hypothesis. A shortening of the intermolt period has been reported for juvenile crabs chronically exposed to copper, although whether this effect was due to an endocrine-disrupting action was not determined (López Greco et al., 2001).

The possibility that heavy metals act as EDC in regard to crustacean molting has not been explored as often as it has with organic compounds. Cadmium has been reported to be taken up by several crustacean species through calcium channels or specific transporting proteins for calcium (Rainbow, 1988; Bondgaard and Bjerregaard, 2005; Norum et al., 2005). Rodríguez Moreno et al. (2003) have reported an inhibition of molting in adults of the crab *Chasmagnathus granulatus* exposed to cadmium from the beginning of premolt. Firstly, the possibility of the inhibition of calcium transport by cadmium, therefore interfering with the normal resorption of the exoskeleton during premolt, was tested and refuted by the authors, as was also the case in a similar study made on crabs throughout their entire molt cycle (Norum et al., 2005). Moreover, since the *C. granulatus* used were eyestalk-ablated, the possibility of an effect of the cadmium on the eyestalk neurohormones was eliminated. Besides, those crabs whose molting was inhibited by cadmium were arrested at the "D_1'' premolt stage, just before the ecdysteroid peak that is needed for ecdysis to occur. Exposure to cadmium beginning after the ecdysteroid peak took place, did not affect molting. These results strongly suggest the possibility that cadmium inhibits molting by inhibiting the secretion of ecdysone. Therefore, whether the inhibition of molting caused by cadmium exposure is due to the blocking of calcium channels in the Y-organs needs to be investigated.

The sesquiterpenoid MF is another hormone with possible roles in crustacean molting (Homola and Chang, 1997). In this regard, Olmstead and LeBlanc (2001) found a decreased frequency of molting in *D. magna* exposed to nanomolar concentrations of methoprene, an agonist of the juvenile hormone of insects, that is used as a growth-regulating insecticide, with several mechanisms involving either agonism or antagonism of juvenoid crustacean receptors being proposed. Inhibition of larval development of copepods by the active juvenile hormone of insects (JHIII) has been also reported (Andersen et al., 2001). However, molting of adult crayfish has been induced by the administration of JHIII (Rodríguez et al., 2002b). Sesquiterpenoid-related compounds have been reported to act as EDCs in insects, leading to molting inhibition (Maymó et al., 1999).

As a general rule, somatic growth is normally correlated with molting, body weight significantly increasing when the animal molts. However, both qualitative and quantitative changes take place during the intermolt period. These changes involve to a large degree the synthesis of new proteins. Decreased somatic growth with no change in the length of the molt cycle has been reported for *D. magna* after exposure to the antiandrogen cyproterone (LeBlanc and McLachlan, 1999), and the surfactant cetyltrimethylammonium bromide and the heavy metals cadmium and copper (Knops et al., 2001). Although it has not been investigated, the observed reduced growth could be caused by altering the endocrine control of the anabolic processes and, in this respect, MF could be one of the hormones involved (Homola and Chang, 1997).

As was suggested by Barata et al. (2004), several pollutants, especially heavy metals, are able to inhibit food intake by small

crustaceans, such as cladocerans and copepods. Such pollutants could be thus reducing the growth rate merely by reducing the energy acquisition and/or by increasing the energy demand associated with the stress caused by these pollutants. Before attributing an endocrine-disrupting nature to any agent that causes reduced growth, it is necessary to eliminate these mentioned possibilities, together with establishing, as directly as possible, interference by the studied pollutant with a specific step in the regulatory mechanisms controlled by the growth hormones. This rationale is also valid for any other hormonally controlled process, such as reproduction or metabolism. For example, if only fecundity was evaluated, more evidence would be needed to conclude that the inhibiting effect of a certain pollutant was due to endocrine disruption, since its effect could be due to only a lethal effect on the eggs, or to direct inhibition of the enzymes involved in vitellin synthesis.

4. Effects on reproduction

The influence of EDCs on a variety of reproductive endpoints has also been explored to assess whether crustaceans are susceptible to the same types of effects as those reported for vertebrates and other invertebrate taxa. Forty days of exposure of neonate (<12 h old) *D. magna* to the synthetic estrogen DES or to the estrogenic pesticide endosulfan did not produce a significant change in the percentage of males produced (Zou and Fingerman, 1997a). However, when daphnids of both sexes (<24 h old) were exposed to DES, the juvenoid analog methoprene or the vertebrate androgen androstenedione, specific effects on development of secondary sexual characters were noted, i.e., increased size of the abdominal process in females by DES and methoprene, and longer first antennae in males exposed to androstenedione (Olmstead and LeBlanc, 2000). These results suggest sex-specific interference by these EDCs on sexual development of crustaceans.

Sex determination of daphnids takes place during a critical period of about 1 h before egg deposition, although evidence of plasticity in sex determination during embryonic development has been provided (Zou and Fingerman, 2003, for review). Eggs produced by parthenogenesis will differentiate into either females or males depending on environmental conditions. If these are unfavorable (extreme temperature, no food, crowding), production of males is stimulated in order to allow sexual reproduction and subsequent production of resting eggs that are able to survive until a favorable environment is reestablished. Similarly, Peterson et al. (2001) found a higher incidence of all-female broods with *Daphnia pulex* exposed to methoprene, while a higher incidence of all-male broods was found when egg-bearing females were exposed to 20-hydroxyecdysone. In addition to the endocrine disruption involved, these results also suggest that sex determination of daphnids is mediated by more than a single hormone, in this case by the endogenous hormones 20-hydroxyecdysone and MF. More recently, induction of male production by MF was reported for *D. magna* (Rider et al., 2005). *D. magna* is certainly a very suitable test species for evaluating in vivo the effect of a wide variety of chemicals on male sex determination (Wan et al., 2005). Dodson et al. (1999) had earlier reported a shift in sex determination toward males, when egg-bearing *Daphnia pulicaria* females were exposed to atrazine (0.5 mg/L) during the entire embryonic development. Atrazine is known to be an EDC in vertebrates, acting through the enzyme aromatase, which catalyzes the conversion of testosterone to estradiol. It is unknown if a similar mode of action occurs in daphnids; but, regardless of its mode of action, it should be stressed that the ecological imbalance of males being produced in the wrong season commonly occurs in aquatic environments where the herbicide atrazine is present (Dodson et al., 1999).

Several estrogenic compounds have been reported to have a low toxicity toward the copepod *T. battagliai*. Hence, estrone, 17β-estradiol and 17α-ethynylestradiol showed NOECs (no observed effect concentrations) with respect to both survival and reproduction at least two orders of magnitude higher than that of 20-hydroxyecdysone (Hutchinson et al., 1999). Furthermore, the sex ratio did not change. Likewise, with the same species exposed to nonylphenol, no change in the sex ratio was observed (Bechmann, 1999). However, nonylphenol caused significant mortality at 62 μg/L, so that only 6% survived to maturity. Nevertheless, the survivors were able to produce enough offspring to increase the population size. Stimulation of the reproductive output was also reported for the copepod *A. tonsa* exposed to 20–23 μg/L of 17β-estradiol or bisphenol A, while no effect was observed with a similar concentration of 2,3-dichlorophenol, a non-estrogenic compound used as a negative control (Andersen et al., 1999).

Amphipods have been also used in several studies for testing the effects of EDCs on crustacean reproduction. Brown et al. (1999) exposed the freshwater amphipod *Gammarus pulex* to the xenoestrogen nonylphenol, and found both a lower survival and a higher fertility (as was mentioned above for the effects of several estrogenic compounds on copepods). Although the sex ratio was not affected, a longer size of the second antennae of males was seen; since this is a secondary sexual character with reproductive value, the authors hypothesized a possible effect of nonylphenol on the androgenic gland. Watts et al. (2002) found a higher sex ratio in *G. pulex* exposed to 17α-ethynylestradiol, at concentrations as low as 0.1 μg/L. Since the relative number of females had increased there was a concomitant increase in the population size as a result of the exposure to this xenoestrogen. Despite these examples of increased fertility caused by exposure to estrogenic compounds, a pathological condition of the ovary was reported for *G. pulex* sampled at river sites where the natural steroids 17β-estradiol and estrone had been detected, together with male fish producing vitellogenin, a typical biomarker of exposure to estrogenic substances (Gross et al., 2001). However, since no purified compounds were tested, it is not possible to be certain about the cause of the pathologies observed.

There have also been some studies on the effects of heavy metals on the reproduction of decapod crustaceans. Rodríguez et al. (2000) found that ovarian growth in the fiddler crab, *U. pugilator*, exposed to cadmium for two weeks was inhibited. This inhibition was seen only in intact crabs, but not in eyestalkless ones, suggesting an effect of cadmium on the secretion

of an eyestalk hormone that regulates ovarian growth. The authors, therefore, hypothesized that cadmium caused increased secretion of the inhibiting hormone, GIH. In an in vitro experiment, isolated pieces of ovary incubated together with thoracic ganglion or eyestalk tissue (ET), inhibition was apparent only when cadmium was added to the vials containing ovary and ET, thereby supporting the hypothesis of the authors.

Medesani et al. (2004a) evaluated the effects of both cadmium and copper on ovarian growth in the estuarine crab *C. granulatus*. Contrary to the results obtained with *U. pugilator*, both heavy metals only produced inhibition of ovarian growth in eyestalkless crabs. In vitro assays were carried out in order to test several hypotheses about the possible causes of these results observed in vivo. Ovary isolates co-incubated with thoracic ganglion did not show any change when cadmium or copper was added to the vials, but decreased ^{3}H-leucine incorporation into ovarian proteins was observed when either of these heavy metals was added to the incubation medium which was supplemented with MF or 17α-hydroxyprogesterone. These results indicate that these heavy metals interfered with one or more steps in the transduction pathway of those hormones at the ovarian level, thereby explaining the inhibition observed in vivo on the eyestalkless crabs. Although the same inhibition would be also occurring in intact crabs, a second inhibition taking place on GIH secretion, suggested by the results obtained in vitro, would be compensating for the former, therefore explaining the absence of an apparent effect in the exposed intact crabs (Medesani et al., 2004a).

5. Effects on glycemia and other physiological variables

Relatively few studies have been done during the last decade on the disruption of endocrine-mediated processes in crustaceans other than growth and reproduction. Hyperglycemic responses in several crustacean species exposed to different kinds of pollutants, including some pesticides, hydrocarbons and heavy metals, have been reported (Fingerman et al., 1998). However, it should be stressed that an observation of an increased hemolymphatic level of glucose alone does not necessarily prove there was a disruptive effect on the endocrine system. Since CHH is released in order to raise glycemia as an adaptive response to several stimuli (such as emersion, starvation, critical temperatures and others), this hormone has been proposed as functioning as a crustacean stress hormone (Chang et al., 1999). Therefore, studies on crustacean glycemia should take into account this physiological stress factor.

Reddy et al. (1994, 1996) have reported hyperglycemia in intact crayfish and crabs exposed to cadmium or naphthalene, but not in eyestalkless ones. Moreover, Reddy et al. (1996) using intact, fiddler crabs, *U. pugilator*, assayed the CHH content of eyestalks taken from crabs exposed previously to cadmium or naphthalene. Injection of eyestalk extracts from cadmium-exposed animals produced a significantly lower hyperglycemia than those prepared from control crabs maintained in clean water, while the extracts from naphthalene-exposed crabs produced a higher hyperglycemic response than that of the control. These results suggest different modes of action of both pollutants: while cadmium could be inhibiting the synthesis of CHH, naphthalene would be increasing this synthesis. More recently, Lorenzon et al. (2000) found that several heavy metals, including mercury, cadmium and copper, induced hyperglycemia in the shrimp *Palaemon elegans*. As a general trend, they found a hyperglycemic response, but it was to be dependent on the heavy metal assayed, the concentration used and the time of exposure. Mercury, cadmium and copper were the pollutants causing hyperglycemia at lower concentrations. Of all the concentrations tested the essential metals copper and zinc produced hyperglycemia, while with mercury, cadmium and lead only the intermediate concentrations assayed had an effect. As for the time of exposure, in all cases glycemia peaked at 3 h after the beginning of exposure, returning to normal values after 8 h in most cases.

Medesani et al. (2001, 2004b) studied the subchronic effects of both cadmium and copper on the glycemia of the crab *C. granulatus*. In the initial study (Medesani et al., 2001), they found a hypoglycemic response in intact crabs, but not in eyestalkless ones, after two weeks of exposure to cadmium or copper. It should be noted that the exposure time maintained in this later study was consistently higher than that employed in the studies mentioned before, where a hyperglycemic response was reported. As in most toxicological assays, longer times of exposure strongly influence sublethal responses. In addition, the secretion of CHH as a stress hormone (see above) would also depend on the phase of the stress syndrome that is taking place (Mayer et al., 1992). According to Medesani et al. (2001), the hypoglycemic response was dose-dependent, copper producing a detectable effect at a concentration 2.5-fold lower than that of cadmium. To evaluate any possible effect on hormone synthesis, the eyestalk CHH content of exposed animals was determined as described above for *U. pugilator*. However, no difference in CHH activity was found between the exposed and control animals. These results suggest that the observed hypoglycemia was due to the inhibition of CHH secretion in the eyestalks. However, the possibility that cadmium or copper interfered with the CHH at target organs could not be eliminated or verified by this experiment. Therefore, to test this possibility, an additional experiment was conducted (Medesani et al., 2004b), that involved CHH injection (32 pmol/crab, purified from *Cancer pagurus*) after a 2-week exposure period to each of these heavy metals. Injection of CHH was able to completely reverse the hypoglycemia caused by cadmium, discarding any possible interference of cadmium on target tissues. But, in the case of copper, a partial reversal was seen, indicating a possible interference of this heavy metal with the transductional pathway of CHH.

The effects of pollutants on the endocrine control of pigment migration have been previously summarized by Fingerman et al. (1998). No new contribution to this topic has been published. In brief, an inhibition of the pigment dispersion in black chromatophores of *U. pugilator* due to exposure to the polychlorinated biphenyl Aroclor1242 and naphthalene was seen, probably due to an inhibition of the release of the black pigment dispersing hormone (BPDH) from the sinus gland. Cadmium produced a similar effect on these black chromatophores. Cadmium also

decreased the ability of the distal retinal pigment to migrate toward the fully light adapted position, presumably by also interfering with the endocrine control of that process.

6. Ecotoxicological relevance

Although no conclusive evidence was reported in field studies, some results clearly indicate that an endocrine disruption of sexual differentiation has been occurring in some crustacean populations. For instance, up to 93% of harpaticoid copepods sampled near the major sewage discharge of Edinburgh showed intersexuality, a condition extremely rare in this group of organisms (Moore and Stevenson, 1991). The incidence of intersexuality was also significantly augmented in marine amphipods from polluted sites of East Scotland (Ford et al., 2004). Moreover, a fine morphological analysis of amphipod males, based on the gnathopod size, also indicates that those males taken from polluted sites and a priori identified as normal (according to the presence of genital papillae), resembled intersex males rather than normal males from reference sites. Since gnathopod development is under the control of the androgenic gland, a demasculinization process seems to be occurring (Ford et al., 2004). On the other hand, feminization was observed in several fish species from the United Kingdom estuaries, but no such effect was observed on crabs from the same estuaries. In the laboratory, estrogenic compounds were able to induce the production of vitellogenin in those fish species, while crustaceans (crabs and shrimps) did not produce vitellin in response to estrogens (Matthiessen et al., 2002).

An important aspect concerning the ecological relevance of the EDCs studies on crustaceans is the possibility of establishing useful biomarkers. In this respect, sensitivity and specificity are desirable properties for a useful biomarker (Hugget et al., 1992). However, only relatively few of the laboratory studies have clearly shown that a significant endocrine disruption occurs at concentrations that can be found in aquatic environments. Despite the fact that these laboratory studies can be used as potential predictors of environmental risk of a certain pollutant, studies that report results at relatively high concentration are mostly aimed at elucidating the mechanism of action of EDC candidates. When establishing biomarkers for EDCs, it is highly desirable that the concentrations that cause endocrine disruption be lower than those having a lethal effect on any stage of the life cycle of the studied species (Barata et al., 2004).

Understanding how any EDC affects a particular endocrine-mediated process is essential to further understand how several EDCs can interact to alter a complex physiological process (i.e., reproduction and growth), regulated in most cases by several interrelated hormones that exert their functions in complex physiological systems. From an ecotoxicological point of view, there is need to evaluate the possibility of a certain compound acting in nature as an EDC at a concentration much lower than that of the compound assayed alone in the laboratory, especially if synergetic interactions are occurring in the environment. A study of the mechanisms of action is intimately related to the need to understand the specificity of action of EDCs and their structure–activity relationships (Schultz and Cronin, 2003), both items being of high ecotoxicological value. The evidence currently available for crustaceans is not yet sufficient for an in depth understanding of EDCs and their effects on those animals.

Sentinel species is another topic to be seriously considered. From these species, biomarkers need to be selected and continuously monitored. Some basic characteristics of sentinel species have been defined (Hugget et al., 1992; Sheffield et al., 1998; Matthiessen et al., 1999): they can be sampled at polluted locations as well as in control, unpolluted sites, their biology and seasonal changes should be well known, and preferably they should be ecologically important. Additionally, some others requirements for sentinel species to be used for evaluating the ecological effects of EDCs have been proposed (Taylor et al., 1999; Depledge and Billinghurst, 1999; Matthiessen et al., 1999): the organism should be relatively insensitive to conventional (non-EDCs) pollutants, be readily cultured in the laboratory, have sexual reproduction and preferably sexual dimorphism, have rapid generation times, and have a relatively well-known endocrine system.

Selection of a crustacean species that perfectly fills all the above mentioned requirements is not an easy task. For instance, daphnids have short generation times and they are easily cultured, but they are very sensitive to a wide range of pollutants and their endocrinology is not well known. A similar profile can be assigned to mysid shrimps, as potential test organisms for evaluating EDCs (Verslycke et al., 2004). Compared to daphnids, though, mysid species have a very cosmopolitan distribution; therefore they are useful in monitoring either freshwater, estuarine or marine environments. A reliable ELISA assay has been recently developed for detecting vitellin in mysid shrimps (Ghekiere et al., 2005), providing a means to easily evaluate the effect of any EDC on vitellogenesis. On the other hand, decapods are relatively resistant to many pollutants; but while their endocrine system is the most extensively studied one among crustaceans, their life cycle is relatively long and their culture in the laboratory is often a complicated operation. Therefore, the selection of more than one crustacean species living in the same environment for monitoring might be necessary to provide the desired information.

7. Concluding remarks

According to several reports, crustacean molting can be inhibited by any one of several organic compounds (including androgenic, estrogenic, as well as antiadrogens and antiestrogens) as well by some heavy metals. However, the endocrine disruption of molting by such compounds has not been still clearly demonstrated. As discussed by Barata et al. (2004) appropriate experimental designs should be devised in order to characterize a certain pollutant as an EDC, since its observed effects could be caused by mechanisms other than the disruption of the endocrine system. In this respect, noteworthy is the development of an in vitro assay to evaluate the effect of pollutants on an ecdysteroid-responsive cell line of *D. melanogaster*. Concerning reproduction, abnormal growth of secondary sexual characters was noted in both sexes of crustaceans, as well as a shift to all-female or all-male broods, after exposure to several

estrogenic compounds, as well as some juvenoids and ecdysteroids. These results strongly suggest an endocrine disruption of the sexual differentiation process. Similarly, a slowed ovarian growth due to heavy metal exposure appears to be related to the disruption of the hormonal control of crustacean reproduction.

From an ecotoxicological point of view, elucidation of the precise modes of action of EDCs would provide a major tool for predicting the effects of a mixture of EDCs, and lead to a more realistic environmental approach to evaluate the effects of EDCs. Assays to determine the toxicity of mixtures of EDCs, as well as the exposure of several species through several generations (especially relevant for long-term processes, such as growth and reproduction), together with assays involving several ecologically related species, are some of the experimental approaches that would extend our knowledge of the toxicity of EDCs and their impact on wild populations.

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