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Bioaccumulation and Metabolic Effects of the Endocrine Disruptor Methoprene in the Lobster, *Homarus americanus*[1]

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SYNOPSIS. Methoprene is a pesticide that acts as a juvenile hormone agonist. Although developed initially against insects, it has since been shown to have toxic effects on larval and adult crustaceans. Methoprene was one of several pesticides applied to the Western Long Island Sound (WLIS) watershed area during the summer of 1999; the other pesticides were malathion, resmethrin, and sumethrin. These pesticides were applied as part of a county-by-county effort to control the mosquito vector of West Nile Virus. Subsequently, the seasonal lobster catches from the WLIS have decreased dramatically. The lethality of the pesticides to lobsters had been unknown. We studied the effects of methoprene while other investigators studied effects of the other pesticides. We questioned whether methoprene, through its effects on larvae, adults or both, could have contributed to this decline. We found that low levels of methoprene had adverse effects on lobster larvae. It was toxic to stage II larvae at 1 ppb. Stage IV larvae were more resistant, but did exhibit significant increases in molt frequency beginning at exposures of 5 ppb. Juvenile lobsters exhibited variations in tissue susceptibility to methoprene: hepatopancreas appeared to be the most vulnerable, reflected by environmental concentrations of methoprene inhibiting almost all protein synthesis in this organ.

Our results indicated that methoprene concentrates in the hepatopancreas, nervous tissue and epidermal cells of the adult lobster. Methoprene altered the synthesis and incorporation of chitoproteins (cuticle proteins) into adult postmolt lobster explant shells. SDS PAGE analyses of adult post–molt shell extracts revealed changes in the synthesis of chitoproteins in the methoprene-treated specimens, suggesting that methoprene affects the normal pathway of lobster cuticle synthesis and the quality of the post-molt shell. Although it is likely that a combination of factors led to the reduced lobster population in WLIS, methoprene may have contributed both by direct toxic effects and by disrupting homeostatic events under endocrine control.

INTRODUCTION

Juvenile hormone (JH) is a regulator of insect development. It modifies the response to the molting hormone, 20-hydroxyecdysone, at the molecular, cellular and organismal level. In larval insects, the presence of JH promotes larval-larvae molts, while its absence results in a pupal or adult molt (Riddiford, 1993, 1996). Methyl farnesoate (MF), synthesized in the mandibular organ of crustaceans, is the unepoxidated equivalent of Juvenile Hormone III (JH III) found in insects. The pesticide methoprene is a JH agonist (Staal, 1986; Wilson, 2004) and also mimics the action of MF in crustaceans (Laufer *et al.*, 1987).

During the warm weather months of 1999, concerns about the spread of the mosquito-borne West Nile Virus led to increased application of pesticide compounds in the New York City and Connecticut area. In mid-September of that year, the region experienced extremely heavy rainfall due to Hurricane Floyd. One month later, lobstermen in Western Long Island Sound (WLIS) began to report sightings of gravid female lobsters dying in the throes of abortive molts. The environmental concentrations of methoprene and of the other pesticides in WLIS during that time are unknown. The combination of circumstances and observations, however, led us to question whether methoprene, known to have been applied to the WLIS watershed, had disrupted the normal hormonal balance in ovigerous female lobsters and triggered molting at inappropriate times. The subsequent decrease since 1999 in the seasonal lobster catches would likewise indicate that one or more harmful events had increased morbidity and mortality among adult lobsters and their offspring. We undertook study of the acute effects of methoprene exposure on the survival of larval lobsters and to determine whether such an exposure would exert changes in the tissues of adult animals. The purpose of this paper is to provide a preliminary communication of our findings, as of January, 2004. A detailed report will be presented elsewhere.

MATERIALS AND METHODS

Culture

All larval experiments were conducted at the Darling Marine Center, University of Maine, Walpole,

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ME. Gravid female lobsters were obtained from local fishermen and maintained in flowing seawater tanks equipped with an outlet screen to retain larvae. As they hatched, larvae were collected with a dip net and transferred to cylindrical tanks (0.3 × 1.5 m) equipped with a similar flow-through system. A large airstone at the bottom of each tank provided adequate suspension of the larvae throughout the entire water column. The ambient water temperature was 18°C; all experimental work with larvae was conducted at the same temperature. Larvae were fed live adult brine shrimp twice daily; the photoperiod was 12 L:12 D. Developmental stages of larvae were identified according to Factor (1995).

Acute exposure studies

All solutions for larval exposure studies were prepared using filtered seawater. S-Methoprene (Welmark International, Schaumburg, IL) was dissolved and diluted in acetone (10 mM) prior to adding it to seawater; seawater used to maintain control animals contained an equivalent amount of neat acetone. Plastic development trays (Pfaff; B&H Photo, New York, NY) were half filled with various concentrations of methoprene in seawater (see Results) and aerated with a small airstone. Trays were maintained at 18°C under fluorescent light in a walk-in cold room. Plastic tackle boxes with 1 mm holes drilled in the bottom were used for exposure studies: each compartment (4 × 5 cm, with a depth of 3 cm seawater) housed one larval lobster. Thirty larvae were exposed to each concentration of pesticide tested (see Results). For postlarvae (Stage IV), 18 individuals were tested at each concentration. Larvae were fed adult brine shrimp *ad lib.* and were scored daily for molting and survival. Dead animals were removed from the trays immediately. Animals were maintained in the original volume of pesticide laden seawater for 7 days; thereafter animals were changed to normal seawater and the experiment was continued for an additional 7 days.

Bioaccumulation studies

Adult intermolt lobsters were equilibrated in the laboratory for at least 7 days. Individual lobsters (~570 g) were placed in plastic buckets containing 8 liter filtered seawater. Dilutions of methoprene (50 ppb final concentration) were made from a stock solution (5 mM) prepared in acetone. Control animals were treated with an equal volume of acetone in seawater. Exposures were conducted at 18°C for 4 hr. Thereafter, animals were anesthetized by packing in ice, sacrificed, and tissues were dissected and snap frozen in liquid nitrogen and stored at −80°C. Tissue samples were transported to the Pesticide Analysis Laboratory at the University of Georgia, Athens GA, where they were extracted and analyzed for methoprene content by gas chromatography-mass spectrometry (GC/MS). Sample preparation for GC-MS was essentially as described by Reed *et al.* (1977). Briefly, tissues (5 g) were homogenized in Na_2SO_4 (50 g) and ethyl acetate (300 ml) for 1 min in a motorized blender. After passage through glass fiber filter paper, the filtrate was concentrated using a rotary vacuum evaporator. The residue was re-dissolved in ethyl acetate: toluene (3:1), vortexed and clarified. The supernatant was defatted by gel permeation chromatography on BioBeads SX-3 (100–200 mesh; Bio-Rad); the eluting solvent was ethyl acetate: toluene (3:1). The sample was further purified by Fluorisil chromatography (serial elution with 6%, 15%, and 50% ether in hexane); material in the 15% fraction was turboevaporated and redissolved in 1 ml methylene chloride. Internal calibration standards were added and the extract was analyzed by gas chromatography-mass spectrometry as described by Noakes *et al.* (1999); the MS instrument was operated in selected ion monitoring (SIM) mode. Gas chromatographic conditions: column: RTX-5M5 col (Restec, Inc.) 30 m by 0.25 mm ID megabore capillary column; oven temperature program: initial temperature = 70°C, initial hold 2 min; temperature programmed to increase at 20°C per minute to 210°C with a final hold of 10 min. Under these conditions, methoprene had a retention time of 29 min, phenanthrene d-10 retention time = 25.5 min; chrysene d-12 retention time = 33.9 min. Data were expressed as parts per million. The methoprene minimal detection limit varied dependent on sample size but was approximately 0.05 ppm (wet weight). A total of five animals have been exposed, dissected, extracted and analyzed thus far; here, we report representative data from a single animal.

In vivo *exposure studies*

Post-molt juvenile lobsters (3 cm carapace length) were exposed to 50 ppb methoprene in sterile seawater (1 liter) for 3 hr; controls were held in seawater only. Each animal was then injected through the dorsal carapace with 0.25 ml seawater containing 0.25 mCi Tran-^{35}S-Label (ICN Radiochemicals, Irvine, CA). The injection site was sealed with a drop of warm 1% agarose in seawater and the animals were held for 24 hr at 18°C. Animals were anesthetized and tissues dissected and placed on ice. As part of the preliminary studies presented here, a total of two animals have been exposed, dissected, extracted and analyzed thus far. Additional replicate experiments are underway.

In vitro *incorporation of ^{3}H D-glucosamine by explant cultures*

Postmolt juvenile lobsters were sacrificed 18 hr after ecdysis (molt stage A2) and the carapace was dissected on ice. After removal of muscle and connective tissue, the carapace was cut into 0.5 × 1 cm strips. About 4 strips were placed in each well of a 6-well plate and covered with 3 ml of explant culture media: Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 5% glucose, 0.49 M sodium chloride, 10% fetal bovine serum, and ABAM (penicillin/streptomycin/amphotericin B). After addition of methoprene (final concentration = 25 ppb) to experimental cultures or acetone carrier vehicle to controls, the samples were

pre-incubated at 18°C for 4 hours. After addition of ^{3}H D-glucosamine (50 μCi/ml; PerkinElmer Life Sciences; Specific activity = 30 Ci/mmol) to each well, the samples were incubated for an additional 16 hr. Afterward, epithelial tissue was removed from the cuticle with a plastic scraper. Samples of shell and epithelial tissue from control or methoprene treated wells were pooled, extracted and processed as described below. In this way, each individual postmolt animal served as its own control.

Tissue homogenization, differential centrifugation and extraction

Each tissue was homogenized with a Potter-Elvejhem homogenizer fitted with a Teflon plunger. Samples were homogenized ten strokes at 50% maximal rpm setting in Homogenization buffer: 20 mM Tris, pH 7.8, containing 0.4 M NaCl, 10 mM $MgCl_2$, 0.2 mM phenylmethanesulfonylchloride and protease inhibitor cocktail (Calbiochem). Shell samples were pre-extracted with 0.5 M EDTA, pH 7, containing PMSF and protease inhibitor cocktail for 12 hr at 18 °C. Homogenates were centrifuged (500 × g for 15 min) to remove cell debris and nuclei. The supernatant was centrifuged at 5,500 × g (15 min) to remove mitochondria and finally at 30,000 × g (45 min) to obtain a crude microsomal fraction (''16Kp'') and cytosol (16Ks). The latter fraction was dialyzed against distilled water using 12 kDa cutoff dialysis membranes.

The 5,500 × g and 16Kp pellets were extracted with 8 M urea containing 0.2% dithiothreitol and PMSF. After centrifugation (10,000 × g for 15 min) the urea soluble supernatant was removed and dialyzed. After washing with 10 mM Tris, pH 7.4, the crude microsomes were collected by centrifugation (30,000 × g for 45 min). The microsomal pellet was then extracted with boiling 2% SDS in 10 mM Tris buffer, pH 7.4 for 5 min. The samples were filtered through Nytex screen and the residue was washed sequentially with water, ethanol and acetone. The final shell residue was air dried at room temperature to constant weight. Radioactivity in all fractions was measured and expressed as cpm/mg sample.

SDS-PAGE procedures

Samples of control and pesticide treated fractions were prepared for SDS-PAGE by boiling in 10 mM Tris, pH 7.0 containing 2% SDS, 15% glycerol, 0.001% brom phenolblue and 0.2% dithiothreitol for 3 min. Samples (15 μl) were applied to either precast 4–20% gradient gels (PageR Gold, Cambrex) or 10% acrylamide gels and separated according to Laemmli (1970). At the completion of the experiment, gels were either fixed and stained for total protein with Sypro Ruby or Coomassie Blue or electroblotted to PVDF membranes using a semi-dry technique. The membranes were then blocked with 5% Blotto (Bio-Rad) in Tris buffered saline (TBS) at 4°C overnight. Blots were probed with biotinylated Tomato Lectin (Pierce Chemical Co) followed by detection with Streptavidin-HRP conjugate (Dako). After final washing, the blots were treated with ECL Plus reagent (Amersham) and positive bands detected using Probe Plus X-ray film (Pierce). After exposure for 1–10 min, films were developed using an automated (Xomat) processor.

RESULTS

Stage II larvae

Initial observations of Stage II larvae exposed to environmental concentrations of methoprene had indicated that exposed animals experienced a consistently higher mortality rate than did the controls. Accordingly, Stage II larvae (30 at each concentration) were maintained in seawater trays containing 0.1, 0.5, 1 and 10 ppb methoprene respectively for 72 hr. During that time period, the mortality rates were essentially the same as that of the controls for animals in the 0.1 and 0.5 ppb trays (approx. 14%). In contrast, at 1 ppb methoprene, the mortality rate was 30% and at 10 ppb, the mortality rate was 86% after 72 hr of exposure.

Stage IV larvae

Overall these animals appeared to be more resistant to the effects of methoprene than were the younger larval forms. They did not experience increased rates of mortality when exposed to methoprene. Instead, methoprene accelerated the frequency of molting in these animals.

Eighteen animals were studied at each concentration. During three days of methoprene exposure at 1 ppb concentration, no animals molted; no control animals molted during this time either. At concentrations of 5 ppb and above, however, the number of molts increased with an average of one third of the animals undergoing molts.

At day three, there were no deaths among the animals exposed to methoprene concentrations below 50 ppb. At 50 ppb, two animals suffered molt-related deaths (11% mortality).

Juvenile lobsters

These animals were exposed to 50 ppb methoprene for 3 hr and then injected with Tran^{35}S-Label. Twenty-four hours later, the hepatopancreas, gills and muscle tissue were removed from each animal and mitochondrial, crude microsomal and cytosolic fractions of each tissue were prepared. As assessed by the incorporation of Tran^{35}S-label into microsomal and cytosolic fractions, the hepatopancreatic tissue from exposed animals suffered greater than 90% inhibition of protein synthesis. The gill tissue exhibited a 50% decrease in protein synthesis while muscle tissue was largely unaffected.

Adult studies

As part of this preliminary study, we exposed a total of three adult animals to 50 ppb methoprene for 3 hr. The pesticide was concentrated in the following tis-

TABLE 1. *Bioaccumulation of methoprene in specific tissues after acute exposure of adult lobsters.*

Sample description	Sample weight (g)	Methoprene concentration (ppm)
Calibration standard	30	3.97
Hepatopancreas	10.39	1.55
Gills	7.52	0.14
Epithelial cells	0.81	6.17
Muscle, abdominal	6.3	0.16
Gonad	1.08	5.18
Stomach	7.17	n.d. @ <0.14
Connective tissue	1.40	n.d. @ <0.71
Eyes	0.35	28.83
Heart	0.70	n.d. @ <1.43

As described in Methods, adult lobsters (molt stage C4) were exposed to 50 ppb methoprene at 18°C for 4 h. Animals were removed from exposure medium, briefly drained then anesthetized on ice for 30 min. Thereafter, tissues were dissected and frozen in liquid nitrogen. Samples were weighed, extracted, and analyzed for methoprene content by GC-MS (see Methods). Results are expressed as concentration of methoprene in parts per million in each of the tissues sampled; n.d., non detectable at or less than the value given. See text for details.

sues: hepatopancreas, gonad, epidermis, and neural tissue (ganglia and eye stalk; See Table 1).

Explant studies

The advantage of using the explant method is that control tissue and experimental tissue are derived from the same animal, thereby obviating inter-animal variation in molt stage or metabolic status.

Incorporation of ^{3}H-glucosamine. Total radioactivity incorporated into 18 hr (molt stage A_2) post-molt epithelial cells in the presence of methoprene (''experimental'') was 38% of that incorporated into control epithelium. Incorporation into the cytosolic fraction from the experimental was approximately equal to that of the control. In the 5,500 × g pellet (containing membrane fragments and mitochondria), however, there were marked differences between the 25 ppb treated specimens and the controls. These pellets were first extracted with urea; the experimental contained 52% less radioactivity than control. The urea insoluble residues were then extracted with SDS; the experimental fraction contained 92% less radioactivity than the control.

Incorporation of Tran^{35}S-Label. The shells from methoprene exposed and control explants were sequentially extracted and total non-dialyzable radioactivity in each fraction was determined. The fractions extracted with EDTA were approximately equal. The buffer extracted fraction of the experimental, however, contained 50% less radioactivity than the control, while the urea extracted fractions were approximately equal. The SDS extracted experimental fraction contained 30% less radioactivity than the control.

SDS PAGE analysis

Analysis of solubilized shell fractions from explant cultures by SDS-PAGE indicated subtle alterations in the total protein profile as revealed by Sypro Ruby staining. As shown in Figure 1a, methoprene treatment caused decreased levels of several protein components with molecular weights of 40, 60 and 75 kDa. On the other hand, when Western blots of duplicate gels were probed with Tomato Lectin (TL, which binds chitin oligosaccharides), differences were observed (Fig. 1b), most notably in the buffer soluble fractions. After methoprene treatment, TL positive components were either absent or reduced at 6, 25, 40, 80, 160 and 240 kDa, suggesting a major effect on an early stage in the synthesis pathway (Horst *et al.*, 1993). Increased levels of specific bands (40, 80 and 160 kDa) were observed in the urea soluble fraction. The SDS soluble fraction appears to have minor quantitative changes as a result of methoprene treatment.

Analysis of the cellular layer of the integument (which is predominantly epithelial cells) by 10% SDS PAGE showed marked differences in the total protein profiles of all extracted fractions (Fig. 2a; changes indicated by stars). Major protein alterations were observed at 45, 100 and 150 kDa. When Western blots of duplicate gels were probed with TL, we again observed alterations in TL positive proteins in the specimens (Fig. 2b). In the cytosolic fraction (lanes 1,2), methoprene treatment caused a shift of TL positive bands from high molecular weight (150–250 kDa) to low molecular weight components (20–40 kDa). SDS extracts of the 16,000 rpm pellet (lanes 5,6) showed decreased levels of 70 kDa material and increased 45 kDa material as a result of methoprene treatment. Analysis of the fractions of the 5,500 × g pellet extracted with urea and then with SDS showed remarkable alterations, but of opposite patterns. The urea soluble fraction showed marked increases in TL positive bands at 120 and 130 kDa as well as clusters of less intense bands at 90, 60, 45 and 36 kDa. By contrast, the SDS soluble material contained fewer bands following methoprene treatment in the higher molecular weight range (60–100 kDa).

DISCUSSION

The effects of JH and JH analogs in insects have been studied extensively (Wyatt and Davey, 1996; Riddiford, 1996; Hammock and Quistad, 1981; Berger *et al.*, 1992; Jones, 1995). In a 1996 review, Riddiford noted that JH allows molting in response to ecdysteroids but alters the switch of gene expression necessary for insect metamorphosis. Methoprene, acting as a JH agonist, has been shown to disrupt the metamorphic reorganization of the insect central nervous system, the salivary glands and the musculature (Restifo and Wilson, 1998). Other investigations indicated toxicity of methoprene to crustacean larvae. Templeton and Laufer (1983) found that methropene at concentrations known to interrupt insect development also interfered with the larval development of *Daphnia magna*. Other investigators found that environmental concentrations of methoprene affected molting, fecundity and the production of male off-

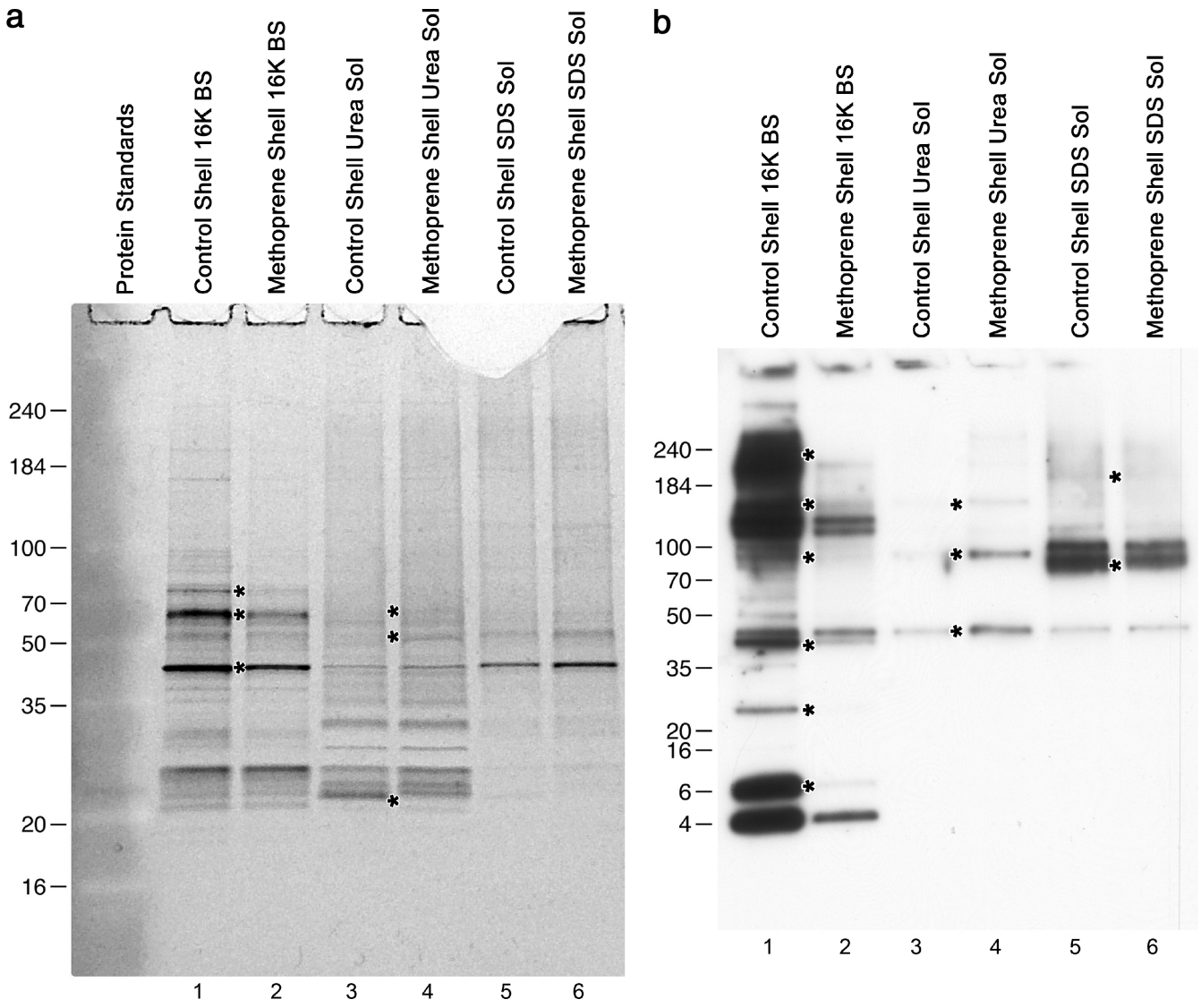

FIG. 1. a. Gradient SDS-PAGE analysis of solubilized fractions from post-molt lobster cuticle. Experimental explant cultures were exposed to 25 ppb methoprene (lanes 2, 4 and 6); control explants are shown in lanes 1, 3 and 5, as described in Materials and Methods. Shell proteins were extracted with buffer (lanes 1 & 2), 8 M urea (lanes 3 & 4) and finally boiled in 2% SDS (lanes 5 & 6). After electrophoresis, the gel was fixed and stained with Sypro Ruby. Position of prestained molecular weight markers are indicated at left of lane 1; molecular mass is reported as kDa. Changes in protein components caused by methoprene exposure are indicated by dots in the buffer soluble and urea soluble fractions. b. Western blot analysis of solubilized fractions from post molt lobster cuticle. Experimental explant cultures were exposed to 25 ppb methoprene (lanes 2, 4 and 6); control explants are shown in lanes 1, 3, and 5. All explants were extracted and separated by SDS-PAGE; as described in Materials and Methods. Proteins were transferred to PVDF membrane by semi-dry blotting and probed with biotinylated Tomato lectin (TL). Binding was detected with streptavidin-HRP followed by enhanced chemiluminescent detection (ECL); the exposure time was 1 min. Position of prestained molecular weight markers blotted to the PVDF membrane are indicated at left of lane 1; molecular mass is reported as kDa. Changes in TL-positive chitoprotein bands caused by methoprene exposure are indicated by dots in the buffer, urea and SDS soluble fractions.

spring in this species (Olmstead and LeBlanc, 2001*a*, *b*, 2003; Peterson *et al.*, 2001). Similar results have been observed in all life stages of salt marsh copepods (Bircher and Ruber, 1988).

Regarding decapod crustaceans, McKenney and Matthews (1990) showed that concentrations of 1 ppm methoprene were uniformly fatal to the larvae of *Palaemonetes pugio* while 0.1 ppm greatly reduced the number of larvae able to complete metamorphosis. Christiansen *et al.* (1977) reported that 1 ppm methoprene was acutely toxic to the larvae of *Rhithropanopeus harrisii*, but these investigators did not find harmful effects at 0.1 ppm methoprene under normal conditions of temperature and salinity. Other studies, however, revealed that this lower concentration did produce adverse effects if coupled with sub-optimal temperature and salinity (Payen and Costlow, 1977; McKenney and Matthews, 1990; Celestial and McKenney, 1994). Additional work by McKenney and Celestial (1993) on larval *Palaemonetes pugio* showed

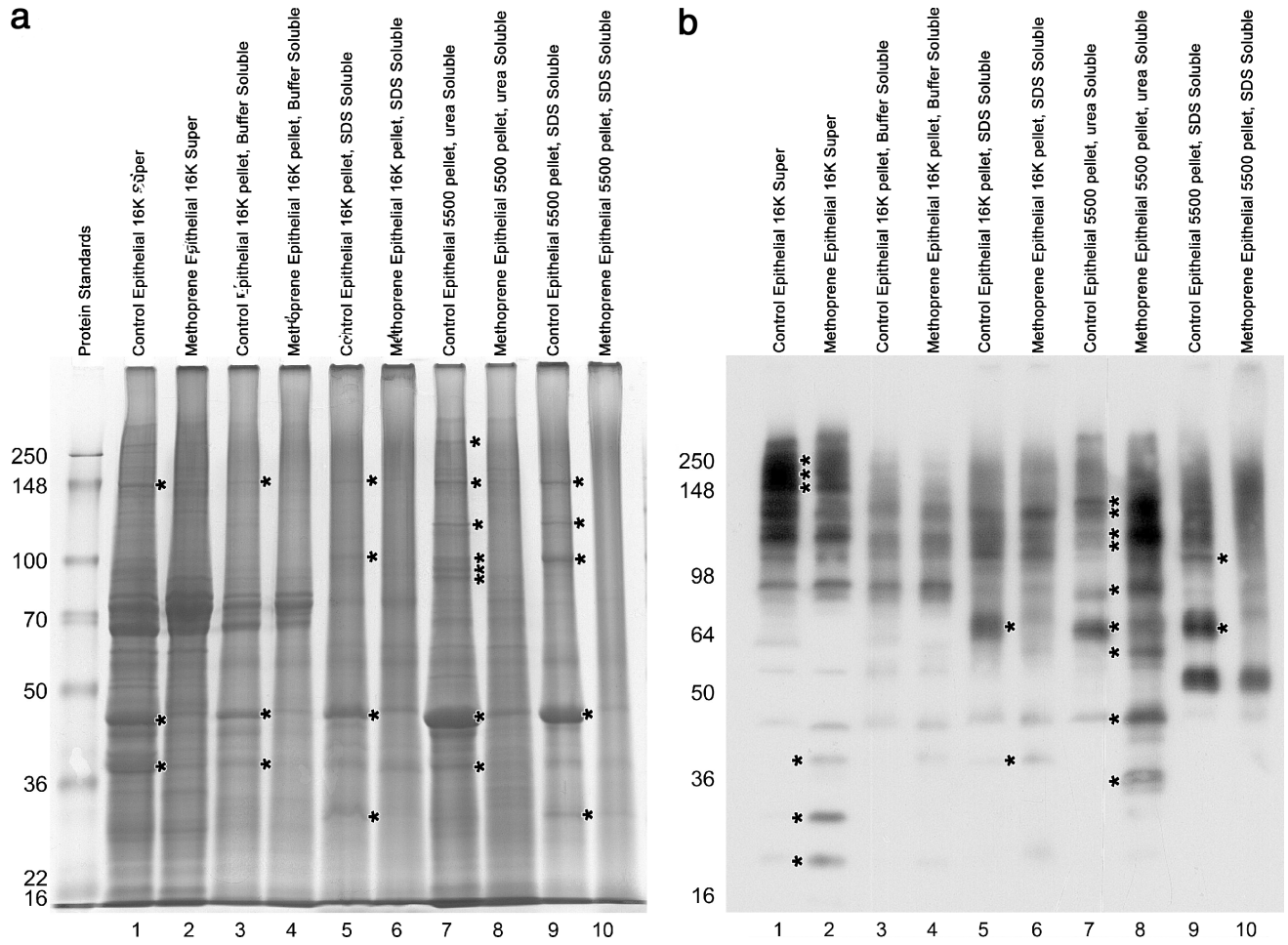

FIG. 2. a. 10% SDS-PAGE analysis of solubilized fractions from postmolt lobster cuticular epithelial cells. Experimental explant cultures were exposed to 25 ppb methoprene (lanes 2, 4, 6, 8, & 10); control explants are shown in lanes 1, 3, 5, 7 & 9. After homogenization of cells, low speed (5,500p) and high speed pellets (16Kp) were obtained and proteins were extracted with buffer, 8 M urea and boiling 2% SDS as described in Methods. Samples were subjected to electrophoresis on 10% gels, fixed and stained with colloidal Coomassie Blue. Samples are identified by lane in the figure. For details, see Methods. The position of prestained molecular weight markers are indicated at left of lane 1; molecular mass is reported as kDa. Changes in the protein profiles of each sample caused by methoprene exposure are indicated by stars. b. Western blot analysis of solubilized fractions from post-molt lobster cuticular epithelial cells. Samples were prepared and separated on 10% gels as described for Figure 3. After electrophoresis, proteins were transferred to PVDF membrane by semi-dry blotting and probed with biotinylated Tomato Lectin (TL). Binding was detected with streptavidin-HRP followed by enhanced chemiluminescent detection (ECL); the exposure time was 5 sec. Lane identities are described in Figure 2a legend. Position of prestained molecular weight markers blotted to the PVDF membrane are indicated at left of lane 1; molecular mass is reported as kDa. Changes in the protein profiles of each sample caused by methoprene exposure are indicated by stars.

that 8 ppb methoprene affected growth and inhibited the completion of metamorphosis.

McKenney and Celestial (1996) found that the juvenile forms of the estuarine mysid *Mysidopsis bahia* suffered complete mortality when exposed to methoprene concentrations of 125 ppb and exhibited diminished size and subsequent fecundity when reared in concentrations of methoprene as low as 8 ppb. Chu *et al.* (1997) found an LD_{50} of 0.34 ppm methoprene at 48 hr for the freshwater cladoceran *Moina macrocopa*; they also found that levels of 5–10 ppb actually stimulated reproduction. Ting *et al.* (2000) found that exposure of the naupliar forms of the harpacticoid copepod *Tigriopus californicus* to 10 ppb methoprene resulted in subsequent disruption of mate recognition, a process known to be under endocrine control. Studies by Ahl and Brown (1990) demonstrated delayed ecdysis and molt-related mortality in brine shrimp larvae (*Artemia* sp.) exposed to 0.3 ppm methoprene.

These last investigators also found that methoprene had a stimulatory effect on Na/K ATPase activity in *Artemia* (Ahl and Brown, 1991). They suggested that the mechanism of action involved direct binding of the pesticide to regulatory sites on the enzyme causing changes in conformation. Due to its hydrophobic structure, methoprene could also lodge in membranes and modify the lipid environment adjacent to enzymes leading to altered conformation and therefore activity. Subsequent studies by Lovett *et al.* (2001) in the green

crab *Carcinus maenas* indicated that MF itself plays a role in osmoregulation.

In our studies of the blue crab (*Callinectes sapidus*), we found that methoprene, in keeping with its hydrophobic nature, could penetrate the investment coat of the blue crab embryo and localize in lipovitellin. Exposure of the embryos to environmental concentrations of methoprene resulted in an overall reduction in the number of successful hatchings and in lethargic swimming behavior on the part of the newly hatched survivors (zoea larvae). Moreover, in later larval forms (megalopae), methoprene delayed the molt to the first crab form and resulted in death of 80% of larvae after exposure for 10 days. It was our conclusion that blue crab larvae exposed to methoprene could either die as a direct result of metamorphic disruption or be compromised in their ability to swim such that they were vulnerable to increased predation (Horst and Walker, 1999).

Although we did not observe altered swimming behavior, we did find that low levels of methoprene had adverse effects on lobster larvae. Methoprene concentrations of 1 ppb or higher significantly affected survival for Stage II larvae. Stage IV larvae were more resistant, but did exhibit significant increases in molt frequency beginning at exposures of 5 ppb methoprene.

Our studies of juvenile lobsters revealed variations in tissue susceptibility to the effects of methoprene. The hepatopancreas appears to be the most vulnerable, with environmental concentrations of methoprene inhibiting almost all protein synthesis in this organ. The hepatopancreas is critical to homeostasis in crustaceans, being involved in digestion of food, absorption of nutrients, production of hemolymph proteins and host defense against infectious agents. The *in vivo* consequences of hepatopancreatic compromise could include, therefore, both increased morbidity and mortality of juvenile lobsters.

Although phylogenetically related, insects and crustaceans have many obvious anatomic and physiologic differences. One difference is the necessity of many crustaceans to molt not only during embryonic and larval stages, but also to continue to molt throughout adulthood. Studies by Laufer *et al.* (1987), Chang (1993) and others have indicated that MF influences the crustacean molt in part by increasing the synthesis of the molt-related hormone, ecdysone. Laufer *et al.* (1998) have also shown that MF stimulates ovarian maturation in the crayfish *Procambarus clarkii*.

The increased frequency of molts in the stage IV larvae and the historical observation of berried females dying while attempting to molt raise the possibility that methoprene could be responsible for endocrine disruption in larval and adult lobsters. Other investigators have shown an interplay between MF and ecdysone in other crustaceans. In 1975, Demeusy suggested that large premolt increases in the hemolymph ecdysteroid titer were due to an ecdysiotrophic action of methyl farnesoate. Then, in 1977, Hinsch demonstrated that, during the premolt, the mandibular organ in *Libinia emarginata* undergoes ultrastructural changes indicative of increased synthetic activity. In *Cancer magister*, Tamone and Chang (1993) showed that ecdysteroid synthesis by Y organs is increased both by incubation with mandibular organ-conditioned medium or with methyl farnesoate. If methoprene is acting as an MF analog, it is reasonable to suggest that methoprene could affect the synthesis of ecdysone, and accordingly influence the timing and frequency of molts. Molting is a stressful and vulnerable time for all crustaceans, larval and adult; thus, increased numbers of molts lead to increased periods of vulnerability to predation, trauma and infection. Moreover, our studies also indicate that methoprene has an effect on the quality of the post-molt shell.

Our studies in blue crabs revealed that methoprene interrupted chitin production in the adult postmolt blue crab as evidenced by decreased incorporation of ^{3}H-glucosamine into methoprene-treated explant cuticle. We also found an effect on protein synthesis as shown by diminished incorporation of radiolabeled amino acids into the explant shells (Horst and Walker, 1999). In the present investigation, we observed that methoprene alters the synthesis and incorporation of both buffer and urea soluble chitoproteins into adult postmolt lobster explants. Presumably, these two fractions represent intermediate stages of glycosylation.

In the present study, SDS PAGE analyses of adult post–molt shells revealed changes in chitoprotein expression in the methoprene-treated specimens. These findings suggest that methoprene blocks the normal pathway of crustacean cuticle synthesis (Horst *et al.*, 1993). Crustacean cuticular chitin is a glycoconjugate complex containing chitin oligosaccharide chains covalently attached to protein. Following biosynthesis, precursors may be exocytosed and incorporated into the postmolt cuticle by crosslinking reactions. In our studies on blue crabs, we established that the SDS insoluble residue of the cuticle contained macromolecular chitin (Horst and Walker, 1999). Such material represents the final stage in the crustacean chitin synthesis pathway; its production by blue crab explants was affected by methoprene when experiments were conducted for a prolonged period of time, *e.g.*, 24–48 hr. The present study on lobster explants included only short-term exposures to methoprene wherein the precursor pool proved to be more dramatically affected than was the formation of final product. Long-term exposure studies are planned.

Bioaccumulation of pesticides has been reported in other crustaceans (Kusk, 1996; Bhavan *et al.*, 1997; Bhavan and Geraldine, 2002; Robinson *et al.*, 2002). The pesticides appear to enter with food into the digestive tract, and are absorbed in the hepatopancreas. There is little information on subsequent transfer of pesticides to other tissues in crustaceans. Our results indicate that methoprene concentrates up to 125-fold over the surrounding sea water in the hepatopancreas, gonadal tissue, nervous tissue and epidermal cells of

the adult lobster. Crustacean epidermal cells are the site of chitoprotein synthesis for shell production. The apparent preferential localization of methoprene into epithelial tissue helps explain the alteration in the chitoprotein expression observed in the exposed explants. Additional bioaccumulation data are presented elsewhere (Walker and Horst, 2004).

Because of the time of the year, Stage II and IV lobster larvae as such would not have been present to experience detrimental environmental influences in WLIS immediately after Hurricane Floyd. Their parents, however, would have been. It has been shown that the quality of the maternal environment influences the survival and success of offspring (Waddy *et al.*, 1995). Methoprene, being lipid soluble, would be expected to accumulate on/in organic debris on the sound bottom. Adult lobsters frequently bury themselves in mud, thereby facilitating their exposure to lipophilic pesticides. Moreover, adult lobsters could also suffer chronic systemic methoprene exposure due to slow release of pesticide deposited in lipid-rich tissues. Even if a berried female had not produced eggs at the time of the contamination, methoprene could concentrate in her lipid-rich tissue and then contaminate her eggs through localization in the lipid-rich lipovitellin. Subsequent embryonic and larval forms could suffer direct toxicity as well as endocrine disruption due to methoprene. Although it is likely that a combination of factors led to the reduced lobster population in WLIS, methoprene may well have played a significant part in this economic and environmental calamity.

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