THE EFFECT OF ALKYLPHENOLS ON LOBSTER SHELL HARDENING

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ABSTRACT Alkylphenols, anthropogenic estrogenic endocrine disruptors in vertebrates, have been found in lobsters (Homarus americanus) in New England sites. We hypothesize that alkylphenols interfere in the shell hardening during molting. We used an in vitro cuticle bioassay to investigate the effects of 2 alkylphenolic compounds—2,4-bis-(dimethylbenzyl) phenol (compound 3) and bisphenol A (BPA; 4,4′-dihydroxy-2,2-diphenylpropane, also referred to as 4,4′-(propan-2-ylidene) diphenol)—on tyrosine incorporation during the hardening of new cuticle following lobster molting. During sclerotization, both alkylphenols and cold-temperature responses, such as a reduction in fertility and meiotic disruptions (Calafat et al. 2005, Crain et al. 2007, vom Saal et al. 2007, Meyers et al. 2009). Alkylphenolic contamination may also be a major contributor to the decreased American lobster (Homarus americanus, Milne Edwards) landings in Long Island Sound (LIS). Landings went from 3.7 million lb (1.68 million kg) in 1998 to 0.6 million lb (0.27 million kg) in Connecticut between 2003 and 2006 (Millstone Environmental Laboratory 2008, Laufer et al. 2012) showed that alkylphenols such as compound 3 and BPA are toxic to larvae at relatively low concentrations and interfere with larval metamorphosis. The financial effect of the lobster die-off has been devastating to the local fisheries and economy in the form of job losses as well as financial hardships.

Previously, we had examined the hemolymph and tissues of lobsters from across southern New England waters for the presence of selected alkylphenolic compounds. We detected alkylphenols in varying concentrations from undetectable levels to more than 4 μg/g (n = 737). Thirty-nine percent of the lobsters were contaminated with at least 1 of 4 alkylphenols (Biggers & Laufer 2004, Laufer et al. 2005b, Jacobs et al. 2008, Jacobs et al. 2012). We used a sensitive invertebrate bioassay using metamorphosis of trochophore larvae of a marine annelid, Capitella, which responded within 1 h of treatment, to determine levels of bioactive compounds, demonstrating that the compounds had juvenile hormone activity, similar to the crustacean juvenile hormone methyl farnesoate (Laufer et al. 1987, Biggers & Laufer 1996, Biggers & Laufer 1999, Biggers & Laufer 2004). Juvenile hormones regulate metamorphosis and the quality of molting in insects (Riddiford & Evans 1994). Our studies found that the following compounds previously found in lobster tissues (Jacobs et al. 2008, Jacobs et al. 2012)—2-t-butyl-4-(dimethylbenzyl) phenol, 2,6-bis-(t-butyl)-4-(dimethylbenzyl) phenol (also known...
as MON-0585, a Monsanto mosquito larvicide similar to Methoprene (Sacher 1971), 2,4-bis-(dimethylbenzyl) phenol (compound 3), and 2,4-bis (dimethylbenzyl)-6-t-butylphenol, 4-cumylphenol, and BPA—had juvenile hormone activity in the Capitella bioassay. BPA was 20 times more active than the crustacean juvenile hormone methyl farnesolate (Biggers & Laufer 2004, Laufer et al. 2012).

Molting is an important process of crustacean development during which sclerotization (tanning and cross-linking of protein and chitin) and shell hardening occur. Hypodermal cells underlying the cuticle secrete cuticular proteins into the subcuticular space (Vacca & Fingerman 1975a, Vacca & Fingerman 1975b, Vacca & Fingerman 1983, Sugumaran et al. 1992, Dillaman et al. 2005). During sclerotization, the phenolic amino acid tyrosine is converted to quinones and their derivatives (Sugumaran et al. 1992), which are then oxidized by phenoloxidases (copper-containing enzymes) to become highly reactive intermediates. These intermediates cross-link with histidyl residues of cuticular proteins and chitin, hardening the exoskeletons (Xu et al. 1997) (Fig. 1). For every tyrosine incorporated, as many as 4 protein molecules are bound. This process can be inhibited when phenoloxidase is irreversibly deactivated by phenylthiourea (PTU), a copper chelating agent (Laskin & Piccinini 1986).

We hypothesize that alkylphenols could play a role in crustacean molting, notably affecting sclerotization by interfering with tyrosine incorporation into the cuticle during shell hardening. Here, we investigate whether alkylphenols (compound 3 and BPA) compete with tyrosine incorporation into cuticles that were monitored during and after a molt. After a molt, radioactive-labeled tyrosine (C14-tyrosine) and radioactive-labeled compound 3 (C14-2,4-bis-(dimethylbenzyl) phenol) were used to compete with unlabeled tyrosine and alkylphenols (either compound 3 or BPA) which reduced radioactive compound incorporation into new cuticle. The levels of competition by the alkylphenols as well as tyrosine incorporation into cuticles were monitored during and after a molt. Competition of alkylphenols to compete with incorporation of tyrosine and its derivatives into the new cuticle was measured, and the time dependence of the level of competition after a molt was determined. We also measured effects of in vivo shell hardening using a pressure gauge to determine the number of days required after a molt for the shell to resist 5 lb pressure without deformation. Any changes in shell structure may delay shell hardening. A delay or change in shell structure may facilitate the invasion of lobsters by microorganisms, making them more susceptible to shell disease.

MATERIALS AND METHODS

Animals

Forty-eight lobsters were used in the current study; preliminary studies performed earlier gave consistently similar results but are not reported here. The lobsters used were collected from LIS, Connecticut, Massachusetts, and Rhode Island (see Jacobs et al. 2012). The animals (average weight, 338.6 g; carapace length, 73.9 mm) were maintained in cooled flow-through seawater (18 ± 2°C) in A-frame tanks at the Marine Biological Laboratory in Woods Hole, MA, and in living stream tanks with closed recirculating seawater equipped with aeration and biofilters at the University of Connecticut. The animals were fed 3 times per week with squid or fish. They were eyestalk ablated to induce molting by removing the sinus gland X-organ complex, which produces a molt-inhibiting hormone.

Chemicals

The following chemicals were purchased from Sigma-Aldrich: 2,4-bis-(dimethylbenzyl)phenol, BPA (4,4′-dihydroxy-2,2-diphenylpropane, sometimes referred to as 4,4′-(propan-2-ylidene) diphenol), PTU, and tyrosine. C14-tyrosine and C14-benzoic acid were purchased from MP Biomedicals, Inc. (Irvine, CA). Scintillation fluid, methyl magnesium bromide, phenol, tert-butyl

Figure 1. Biochemical reactions involved in sclerotization and the linking of tyrosine metabolites (Matthews & Van Holde 1990, Xu et al. 1997).
chloride, zinc chloride, and ethanol were purchased from Fisher Chemical. Diazomethane was prepared from Aldrich Diazalid by normal procedures. Salinity was maintained at about 30 ± 2% using Coral Reef Red Sea Salt Mix using a Vitalsine refractometer to adjust salinity.

Synthesis of C14-2,4-Bis-(dimethylbenzyl) Phenol, Compound 3

Diazalid (N-methyl-N-nitroso-p-toluene sulfonamide) was dissolved in dry ether and added slowly to a mixture of KOH pellets, distilled water, and 2-(2-ethoxyethoxy) ethanol at 70°C. After distillation using the Aldrich Chemical Co. Diazomethane Apparatus, we obtained diazomethane. The distilled diazomethane solution was then added to benzoic acid that had been labeled (0.5 μCi/mL) with C14 in the carboxylic carbon (step 1, Fig. 2) and then dissolved in dry ether. After 2 h, the solvent was distilled off under a stream of dry nitrogen to give 5% benzoic acid and 95% labeled methyl benzoate, methyl alpha-C14 benzate (step 2, Fig. 2). Labeled methyl benzoate was then added dropwise to a stirred solution of magnesium bromide and aqueous potassium hydroxide to produce compound 3—2,4-bis-(dimethylbenzyl) phenol—in radioactive form (Fig. 2) (see also Biggers & Laufer 2004).

Radioactive Tyrosine/Alkylphenol Incorporation

For the in vitro culture experiments, we sacrificed lobsters during the molt between 2 h and 8 h after ecdysis, and cut the newly molted cuticle into 0.2–0.3-g pieces. Other samples were taken at 1, 2, and 4 days after a molt. We cultured each cuticle piece in 1 well of a 24-well culture plate with 500 μL Pantin's solution (Pantin 1934) containing 30.65 g NaCl, 0.99 g KCl, 1.82 g CaCl2·2 H2O, 5.04 g MgCl2·6 H2O/L, pH 7.0. Each sample was then exposed to 10 μL radioactively labeled tyrosine (0.1 μCi/mL) or C14-2,4-bis-(dimethylbenzyl) phenol (C14-compound 3; 0.02 μCi/mL) for incorporation studies. In parallel experiments, tissue samples were incubated with a radioactive precursor and one “competitor” (cold tyrosine, cold compound 3, 2,4-bis-(dimethylbenzyl) phenol, BPA, or PTU). Specimens were incubated for 4 h at room temperature, then fixed in 1 mL ethanolic KOH and washed 3 times with Pantin's solution. After separation of cuticle and epidermis, cuticle samples were transferred to scintillation vials with 2 mL scintillation fluid, were left in vials overnight, and were counted the next day.

Injection of lobsters in Vivo

Premolt lobsters were previously eyestalk ablated 10–12 days before injection with either 0.2 mL peanut oil (controls) or 0.2 mL 2 mM compound 3 in peanut oil suspension (experimental). Injections were done weekly until lobsters molted. Injections were performed into the abdomen at the junction of the soft cuticle of the posterior abdominal leg attachments.

Shell Hardness Measurements

Measurements were carried out on the anterior dorsal shells of molted lobsters that were injected with peanut oil (control group) or compound 3 (experimental group). The same dorsal intact carapace area was tilted to subject it to 5 lb pressure by a pressure gauge to determine whether the shell was able to resist shell deformation. Tests were performed using a size 0 cork attached to the Pelouze model K pressure gauge (manufactured by a division of Healthometer, Inc.).

Statistical Analysis

The lobsters were usually acclimated to the laboratory and eyestalk ablated. This means that they were usually maintained for about a month or more until they molted. No geographical difference in the sources of the lobsters was observed or noted. Results are presented as a mean ± SD. A 1-way ANOVA was used to analyze the data statistically. Values were considered significant at P < 0.05, and highly significant at P < 0.01 or more.

RESULTS

C14-Tyrosine Incorporation in the Lobster Cuticle During and After a Molt

C14-tyrosine incorporation occurred in a time-dependent manner, decreasing from 34,800 DPM after a molt to 16,000
DPM at day 1, less than 10,000 DPM by day 2, and 2,200 DPM at 4 days after a molt, for a total decrease of 93.5% (Figs. 3–6) (*P < 0.01 for each day after the molt compared with the day of the molt). This was also observed for the competitors such as alkylphenol (compound 3 (Figs. 3–6) and BPA (Figs. 4 and 5)) and PTU. For example, 30 μM (or 10 μg/mL) alkylphenol treatment after a molt showed 14,100 DPM and decreased to 6,800 DPM by 2 days after a molt (decrease of 51.7%), and finally to 1,600 DPM by 4 days after a molt (a decrease of 88.7%; *P < 0.01).

Cold tyrosine competed significantly with C14-tyrosine incorporation in the cuticle after a molt (*P < 0.01), decreasing C14-tyrosine incorporation to 15,000 DPM at 27.5 μM (5 μg/mL) cold tyrosine in comparison with 34,800 DPM for the untreated (decreased by 56%). Competition was also concentration dependent as 10 μM (55 μg/mL) cold tyrosine decreased C14-tyrosine incorporation in the cuticle by 63.9% (Fig. 3). Similar concentration-dependent inhibitory effects were observed 1, 2, and 4 days after a molt (Figs. 4–6) (*P < 0.01).

As expected, in nonmolting cuticle, untreated and treated cuticles showed no significant C14-tyrosine incorporation (Fig. 7) (*P > 0.05, **P < 0.01).

C14-Tyrosine Incorporation Is Inhibited in Lobster Cuticle by Alkylphenol (Compound 3) Competition

The alkylphenolic compound—2,4-bis-(dimethylbenzyl) phenol (compound 3)—also competed in a concentration-dependent manner with C14-tyrosine incorporation (Figs. 3–6) (*P < 0.01). During a molt and 1 day after a molt, compound 3 exhibited a greater degree of competition than that of cold tyrosine (71% vs. 56% after a molt and 57% vs. 44% for 1 day after a molt; Figs. 3 and 4).
PTU, a phenol oxidase inhibitor, exhibited a concentration-dependent inhibition of C\textsuperscript{14}-tyrosine incorporation in the cuticle ($P < 0.01$ during a molt). PTU's high level of inhibition decreased over time after a molt, but even at 4 days after a molt, inhibition was still high (79% after a molt to 48% at 4 days after a molt; Figs. 3–6). These results support the concept that tyrosine incorporation into the cuticle is phenol oxidase dependent.

Another alkylphenol, BPA, exhibited the greatest level of concentration-dependent inhibition of C\textsuperscript{14}-tyrosine incorporation in the cuticle. At 1 day after a molt, there was a 74% inhibition (or 4,100 DPM in comparison with 16,000 DPM for untreated cuticle; $P < 0.01$), and it did not decrease with time, as the DPM count was similar at 2 days after a molt (Figs. 4 and 5). In comparison, this inhibition is greater than the established phenol oxidase inhibitor PTU. At 1 day after a molt, C\textsuperscript{14}-tyrosine incorporation in the cuticle for the untreated was 16,000 DPM whereas 15-$\mu$M BPA and PTU treatments resulted in 4,100 DPM and 4,800 DPM C\textsuperscript{14}-tyrosine incorporation, respectively. At 2 days after a molt, C\textsuperscript{14}-tyrosine incorporation for the untreated cuticle was 9,700 DPM whereas 15-$\mu$M BPA and PTU treatments resulted in 2,500 DPM and 3,800 DPM, respectively ($P < 0.01$). Similar inhibitions were also observed for 30-$\mu$M BPA (2,500 DPM and 1,000 DPM, respectively, for 1 and 2 days after a molt; $P < 0.01$) and PTU treatments. These results are significant because, for the first time, they establish that BPA is a potent inhibitor of tyrosine incorporation in the cuticle, and its inhibitory activity is greater than that of the known enzyme inhibitor PTU.

C\textsuperscript{14}-\textit{2,4-Bis-(Dimethylbenzyl phenol Incorporation in the Cuticle and the Inhibitory Effects of Competitors During a Molt}

C\textsuperscript{14}-compound 3 was synthesized, and \textit{in vitro} assays were carried out to study uptake and competition of incorporation in the cuticle during a molt. Cold alkylphenol inhibited C\textsuperscript{14}-alkylphenol incorporation significantly ($P < 0.01$) in a concentration-dependent manner (9,500 DPM for untreated to 5,100 DPM and 3,700 DPM for 15-$\mu$M and 30-$\mu$M alkylphenol, or inhibition of 46% and 61%, respectively; Fig. 8). Tyrosine exhibited a 27% and 34.5% inhibition at 15-$\mu$M and 30-$\mu$M, respectively. Last, at 15-$\mu$M and 30-$\mu$M PTU treatments, inhibition of C\textsuperscript{14}-alkylphenol incorporation was at 38% and 53%, respectively ($P < 0.01$; Fig. 8). These results provide further evidence of this alkylphenolic compound incorporation in the cuticle after a molt, supporting the earlier findings of this alkylphenol as a competitor of tyrosine incorporation in the cuticle after a molt.

\textbf{In Vivo Alkylphenol Inhibition of Shell Hardening}

Lobsters were either treated with peanut oil (control) or alkylphenol (compound 3 in peanut oil) and were tested for shell hardening after a molt. A calibrated pressure gauge that exerts 5 lb pressure was used on intact carapace shells of lobsters on various days after a molt. Although the control shells required 7.1 days ($n = 15$) to harden sufficiently to resist deformation by the pressure gauge, the treated shells needed 12.1 days ($n = 9$) to resist the same pressure ($P < 0.01$; Fig. 9). Furthermore, treated lobsters required an extra 17 days to molt, in comparison with their untreated counterparts. These studies show that alkylphenolic compound treatment noticeably inhibited and delayed molting as well as the postmolt-associated shell hardening process in lobsters.

\textbf{DISCUSSION}

We have presented evidence that 2 of the anthropogenic alkylphenols we detected in hemolymph and tissues of field-caught lobsters from all over southern New England, including LIS (Jacobs et al. 2008, Jacobs et al. 2012), interfered with shell hardening in lobsters by competing with tyrosine and its derivatives. This interference occurred during critical early steps of sclerotization (1–4 days). This effect was suggested previously in studies performed by several investigators in other invertebrate organisms (Sacher 1971, Zomer & Lipke 1981, Semensi & Sugumaran 1986). They showed that mosquito larvae treated with another alkylphenolic compound—MON-0585 (2.6 bis-(t-butyl)-4(dimethylbenzylphenol), which we also
found in lobsters (Biggers & Laufer 2004)—experienced 100% mortality resulting from toxicity at metamorphosis at a concentration of 1 ppm. The larvae initiated the molt into the pupal stage, but although normal pupae were dark brown and hardened after cuticle sclerotization, treated larval pupae intermediates remained soft and were white. These results suggested that MON-0585 interfered specifically with metamorphosis, tanning, and cuticle hardening during the molting process.

The amino acid tyrosine is a phenol and plays a key role in the sclerotization (hardening and tanning) of the lobster cuticle (Fig. 1). During cuticle formation, tyrosine is metabolized by the enzyme tyrosinase or phenoloxidases to n-acetyl dopamine and then to a quinone (Sugumaran et al. 1992, Xu et al. 1997). If the tyrosine derivatives are replaced by alkylphenols, which have a more complex structure, there are fewer opportunities for protein or chitin cross-linking, thus weakening the shell.

We found that tyrosine and alkylphenols could incorporate into the new cuticle of lobsters, and that alkylphenols competed with tyrosine during the incorporation. As indicated in Table 1, 5 μg/mL and 10 μg/mL tyrosine are nearly 2 times the equivalent in concentration as that of the alkylphenol (compound 3). The data in Figure 3 suggest that alkylphenols outcompete tyrosine in the shell hardening reaction. This reaction is mostly dependent on the enzymatic activity of phenoloxidase, therefore resulting in less tyrosine incorporation. These data supported our contention that alkylphenolic contamination interferes with shell hardening. We also found that C14-tyrosine did not incorporate into cuticle of nonmolting lobster, suggesting that tyrosine incorporation into lobster cuticle happens mostly during postmolt cuticle formation.

TABLE 1.
Tyrosine, 2,4-bis-(dimethylbenzyl) phenol (alkylphenol), and phenylthiourea equivalents.

<table>
<thead>
<tr>
<th>Tyrosine (μM)</th>
<th>Alkylphenol (μM)</th>
<th>Phenylthiourea (μM)</th>
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<tr>
<td>5 μg/mL</td>
<td>27.5</td>
<td>15</td>
</tr>
<tr>
<td>10 μg/mL</td>
<td>55</td>
<td>30</td>
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More important, these experiments provide clear evidence that alkylphenols, even at half the concentration as that of tyrosine, incorporate into the cuticle during shell hardening at a more competitive rate than tyrosine and its derivatives. These results are significant because, for the first time, they establish that BPA is a potent inhibitor of tyrosine incorporation in the cuticle, and its inhibitory activity is greater than that of the known enzyme inhibitor PTU.

Alkylphenols do not cross-link as successfully as tyrosine and its derivatives. This suggests that alkylphenols are taken up by molting cuticles. Alkylphenol-treated cuticle remains soft for extended periods of time. The possible reason may be that alkylphenol contamination affects the structural integrity of lobster shells. Sacher (1971) also described similar effect on mosquito larval cuticles which were prevented from sclerotizing and melanizing when treated with 1 ppm 2,5-bis-(t-butyl)-4-(dimethylbenzylphenol)—an alkylphenol also known as MON 0585. Pupation and metamorphosis were interrupted in the mosquitoes because the dose was lethal at this stage. We also found (Laufer et al. 2012) that alkylphenols, both compound 3 and BPA, were toxic to larval lobsters before and at metamorphosis, and that they interfered with the normal larval–juvenile transition by resulting in high percentages of intermediates among the few survivors.

The use of radioactive tyrosine and radioactive compound 3, 2,4-bis-(dimethylbenzyl) phenol, shows that these compounds are taken up by the cuticle. The use of nonradioactive competitors, including BPA, shows that these compounds compete with the uptake and incorporation of these radioactive compounds into the shell. The in vivo shell hardening experiment shows that there is a weakening effect of alkylphenols on the actual shell hardening process. Kunkel and Jercinovic (2012) suggest that the carbonate apatite structure of the lobster cuticle, which gives the shell its major strength, is dependent on proper protein cross-linking structures that are apparently disrupted here by substituting alkylphenols for tyrosine. An alteration of shell structure by contaminants such as alkylphenolic pollutants makes the shell weaker and may make the shells more susceptible to microbial invasion, thus possibly contributing to such phenomena as lobster shell disease. Increased susceptibility to shell disease would certainly affect the lobster population negatively, because it has been observed that ovigerous lobsters molt in defense of shell disease, discarding their embryos with their shell (Laufer et al. 2005a, Laufer et al. 2005b).

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LITERATURE CITED


