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Acetyl Cholinesterase Activity and Muscle Contraction in the Sea Urchin Lytechinus variegatus (Lamarck) Following Chronic Phosphate Exposure

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ABSTRACT: The common shallow-water sea urchin Lytechinus variegatus is capable of surviving inorganic phosphate exposures as high as 3.2 mg L^{21} and organic phosphate exposures of 1000 mg L^{21}. Nonetheless, chronic exposure to low, medium, and high-sublethal concentrations of organic phosphate inhibits the muscle enzyme acetyl cholinesterase (AChE), responsible for the break down of the neurotransmitter acetylcholine, as well as inhibiting contractions in the muscles associated with the Aristotle's lantern. AChE activity, measured in both a static enzyme assay and by vesicular staining, displayed concentration-dependent declines of activity in individuals maintained in organic phosphate for 4 weeks. The activity of AChE was not adversely affected by exposure to inorganic phosphate or seawater controls over the same time period. Maximum force of muscle contraction and rates of muscle contraction and relaxation also decreased with chronic exposure to increasing concentrations of organic phosphate. Chronic exposure to inorganic phosphates elicited no response except at the highest concentration, where the maximum force of muscular contraction increased compared to controls. These findings indicate that shallow-water populations of Lytechinus variegatus subjected to organic phosphate pollutants may display impaired muscular activity that is potentially related to the inhibition of the muscle relaxant enzyme AChE, and subsequently muscular overstimulation, and fatigue. # 2010 Wiley Periodicals, Inc. Environ Toxicol 21: 000–000, 2010.

Keywords: phosphate contamination; acetylcholine esterase; enzyme activity; muscle contraction; sea urchin

INTRODUCTION

Phosphate pollutants occur in nearshore marine environments as a result of their seasonal use in agriculture (Pait et al., 1992, Rabalais et al., 2002), urban drainage, and storm water run-off (Miles and Pfeuffer, 1997). Increasing concen-
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trations of inorganic phosphates have been linked recently to increases in algal growth and eutrophication (Justic et al., 1995; Lin et al., 1995; Rabalais et al., 2002; Sylvan et al., 2006; Scavia and Donnelly, 2007). Because the restriction in use of chlorinated pesticides due to their environmental persistence, organic phosphates and carbamates have become the alterative pesticides (Pait et al., 1992; Forget et al., 2003). Organophosphate pesticides are known neurotoxins that affect the breakdown of acetylcholine, the primary neurotransmitter in the sensory, and neuromuscular system of many organisms (Eto, 1974; Payne et al., 1996; Key et al., 2003; Cunha et al., 2005). Inhibition of AChE, an enzyme used in muscle relaxation, results in build-up of

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acetylcholine, which, in turn, causes continuous and excessive muscle stimulation leading to tetany, paralysis, and eventual mortality. There is evidence for the presence and activity of cholinesterases in the nervous tissue of sea urchins (Bacq, 1937; Augustinsson and Gustavson, 1949; Pentreath and Cottrell, 1968; Medes et al., 1970; Pentreath, 1970; Pentreath and Cobb, 1972), and it has been used as a biomarker in environmental studies (Cunha et al., 2005). Exposure to sublethal concentrations of inorganic and organic phosphates is known to adversely influence aspects of feeding, nutrient absorption and allocation, growth, fertilization, development, and immunology in marine invertebrates, including sea urchins (Böttger and McClintock, 2001, 2009; Böttger et al., 2001).

The Gulf of Mexico ranks first among coastal areas of the United States in terms of total square kilometers of estuarine drainage and total water surface area (Lovejoy, 1992). Environmental degradations characterizing the waters and shores of the Gulf of Mexico are a result of anthropogenic pollution, especially within the proximity of estuarine, groundwater, and stormwater drainage areas (Lovejoy, 1992; Rabalais, 1992; USGS, 2001). The application of inorganic and organic phosphates to agricultural land is seasonal and takes place primarily in the spring and summer. Subsequent runoff results in the deposition of significant quantities of phosphate pollutants in drainage areas (Lovejoy, 1992; Rabalais, 1992; Rutkowski et al., 1999) and eventually in shallow bays and nearshore waters of the Gulf of Mexico affecting seegrass distribution (Rutkowski et al., 1999), physiological processes of the sea urchin *Lytechinus variegatus* (Böttger and McClintock, 2001, 2002, 2003; Böttger et al., 2001), and other marine invertebrates (Key et al., 2003).

Here, we report the effects of chronic phosphate exposure on muscle contraction and the activity of the muscle relaxant enzyme acetyl in the sea urchin *Lytechinus variegatus*. This species is abundant (Beddingfield and McClintock, 1999, 2000), widely distributed, and significantly affects seagrass community structure in areas impacted by phosphate pollutants [(Valentine and Heck, 1991; Greenway, 1995; McGlathery, 1995; Macia, 2000; reviewed by Watts et al. (2001)]. Quantifying the effects of phosphate pollutants on muscle physiology is important to understand how impaired muscular activity could negatively affect locomotory and foraging behavior.

MATERIALS AND METHODS

Chemicals

Phosphate pollutants sodium (NaH_2PO_4) and triethyl ($(C_2H_5O)3P(O)$) phosphate were purchased through Sigma-Aldrich Chemicals. For the colorimetric determination of cholinesterase activity, acetylthiocholine iodide (cholinesterase substrate) and $5,5^{0}$-dithiobis-2-nitrobenzic acid (DTNB) were purchased through Sigma Biochemicals and Reagents, and phosphate-buffered saline (reagent grade phosphate buffer) was purchased through FisherBiotech. Muscle tissue used for histological staining was embedded using an OTC compound manufactured by Sakura Finetek USA.

Phosphate Pollutants

Total concentrations of inorganic phosphates in streams entering the northern Gulf of Mexico may attain levels of 3.2 mg L^{21} (Lovejoy, 1992), and ambient concentrations as high as 0.8 mg L^{21} have been found in pristine environments (Rafaelli, personal communication), with EPA limits of 1 mg/L phosphorous in drinking water (Bartenhagen et al., 1994). These baselines were used to select a presumed maximum sublethal concentration of 3.2 mg L^{21} of the inorganic sodium phosphate for our experiments.

Triethyl phosphate, the organic ingredient of a wide range of organophosphorous insecticides, a component in flame retardants and plasticizers, and a resin modifier is known to exhibit the same effects on nerves and muscles as organophosphorous insecticides (Eto, 1974). With a half-life in water of potentially 5.5 years (SIDS Initial Assessment Profile 78-40-0), subacute toxicity of 1000 mg L^{21} (kg) and break down products including inorganic phosphorous and carbon dioxide (Cartwright, personal communication), triethyl phosphate is an ideal representative of organophosphorous insecticides for experimental analysis. The use of organophosphorous pesticides, while common, is a relatively recent phenomenon, and concentrations in the Gulf of Mexico have not yet been extensively studied (Lytle, personal communication). However, triethyl phosphate has been the subject of toxicological research, and concentrations as high as 1000 mg L^{21} have been used in bioassays (Gumbmann et al., 1968). Additional preliminary toxicological studies using logarithmically increasing concentrations ranging from 0 to 1000 g L^{21} (n 5 10 individuals per treatment) lead to the selection of experimental concentrations of 10, 100, and 1000 mg triethyl phosphate per liter seawater for our experiments.

Animal Collection and Maintenance

Lytechinus variegatus of similar size (test diameter 30–50 mm) were collected from Saint Joseph's Bay, a pristine bay in northern Florida. Fifteen individuals were sacrificed immediately to establish baseline measurements for AChE activity, maximum force of muscle contraction, and rates of contraction and relaxation of the Aristotle's lantern. All remaining individuals were maintained in 20-L recirculating sea water systems at ambient field conditions of 188C and 30% salinity. Twenty individuals were chosen at random and placed into each of three different phosphate concentrations of 0.8, 1.6, or 3.2 mg L^{21} sodium phosphate and 10, 100, and 1000 mg L^{21} triethyl phosphate. A control group

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of 20 individuals was maintained in artificial seawater. The individuals in each treatment concentration were subdivided into two recirculating tanks with 10 individuals per tank to avoid crowding and detect possible "tank effects." Maintenance of consistent phosphate concentrations in individual tanks required measuring and adjusting of concentrations on a weekly basis [using a colorimetric assay for inorganic phosphates and spectrophotometrical analysis (APHA, 1988) for organic phosphates]. All treatments were also subjected to partial water changes (10 L) every 48 h and weekly to ensure the stability of the phosphate concentrations across the treatments. Upon completion of a 4-week experimental period, all individuals were sacrificed, and muscles of the Aristotle's lantern were used for further analysis.

Colorimetric Determination of AChE Activity

AChE activity was measured initially and at the end of the 4-week experimental period in the retractor muscle of the Aristotle's lantern using the Gorun et al.'s (1978) modification of the procedure developed by Ellman et al. (1961). Muscles from 10 individuals from each treatment were examined in this colorimetric assay. Tissue samples (100 mg) of each of the muscles were homogenized on ice in 5 mL of a 20 mM phosphate buffer at pH 7.6. A subsample of 20-1L homogenate (containing the enzyme AChE) was combined with 20 1L of 7.5 mM acetyl thiocholine (substrate) and 160 1L of 20 mM phosphate buffer to a final volume of 0.2 mL. The mixture was allowed to react at room temperature (258C) for 30 min, and the reaction was stopped through the addition of 1.8 mL 10 mM DTNB-phosphate-ethanol reagent. Samples were examined spectrophotometrically at 412 nm using a boiled sample of Aristotle's lantern muscle as a control to ensure that any change in absorption was caused by the presence of AChE. Activity was calculated in 1M^{21} mg^{21} tissue min^{21}.

Cholinesterase Staining

Upon dissection, tissues extracted from the retractor muscles of the Aristotle's lantern of five individuals were embedded in OCT compound (embedding medium for frozen tissue specimens), cryostat preserved, and sectioned at 8 1m. Sections were mounted on slides and stained in an incubating solution for nonspecific esterases (Karnovsky and Roots, 1964; Karpati and Engel, 1968; Ringel et al., 1976). Slides were rinsed in distilled water and counterstained with hematoxylin. Vesicles displaying cholinesterase activity stained red/brown. Numbers of partially and fully stained vesicles per micrometer square were counted using a Leitz diplan biocular microscope.

Isometric Muscle Contractions

Upon dissection, the Aristotle's lantern protractor muscle was removed leaving the ends of the muscle attached to a tooth of the Aristotle's lantern and the skeletal test as anchors. The muscle was attached to a glass rod on one end and a force transducer (0.5–10 g) connected to a computer on the other. Muscles were stimulated electrically (0–100 V; 0–20 ms) to generate minimum and maximum strength/duration curves for each treatment and to calculate the rheobase (threshold stimulus intensity at the longest stimulation). Following these standard measurements, continuous stimulations of 20 V, 2 ms, and 10 pulses/sec and 40 V, 1 ms, 10 pulses/sec were chosen for experimental evaluation. Because of their high-rheobase values (mean 5 30.45 V), individuals maintained in the highest organic phosphate concentration did not display any contraction at 20 V, even under continuous stimulation. Therefore, results are only available for this treatment at 40 V. Aristotle's lantern retractor muscles from five individuals treatment 21 were stimulated, and results were recorded using a Virtual Bench Datalogger. Muscular contraction measurements were repeated stimulating the muscles with 0.1 1M acetyl choline (ACh), a known neurotransmitter in invertebrates. Data were examined using Origin 41, and maximum force of contraction at tetanus [(N force mm^{21} cross-sectional area) 3 10^{24}], contraction, and relaxation rates [(N force/s) 3 10^{24}] were compared between treatments.

Statistical Analyses

A one-way ANOVA followed by a Tukey–Kramer multiple comparison test was used to compare AChE activity, levels of cholinesterase staining, maximum force of contraction, and contraction and relaxation rates in experimental and control treatments following the 4-week phosphate exposure period. Before statistical analyses, assessments of the assumptions of normality (Kolmogorov–Smirnov test) and homoscedacity (Spearman-Rank Correlation) were conducted. An arcsine transformation was conducted to normalize the data before statistical analysis.

RESULTS

No tank effects were observed for control and experimental animals separated at random into groups of 10 animals/tank into two tanks/treatment.

AChE Activity

Acetyl cholinesterase (AChE) activity in retractor muscles of the Aristotle's lantern of *Lytechinus variegatus* did not change significantly (P 5 0.571) in individuals maintained in all inorganic phosphate treatments compared to initially sacrificed individuals and those maintained in artificial seawater [Fig. 1(A)]. Muscles of individuals maintained in all F1 three concentrations of organic phosphate displayed

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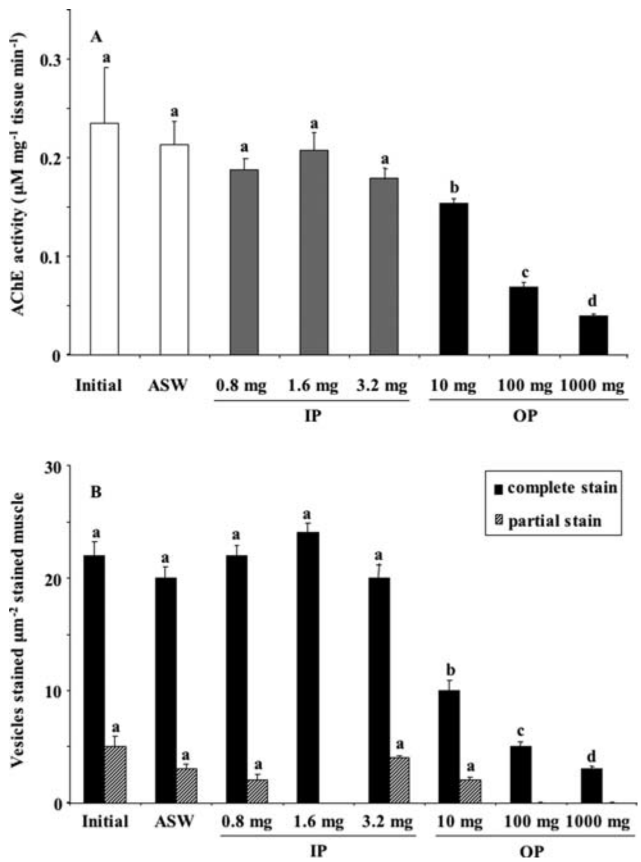

Fig. 1. Acetyl cholinesterase inhibition in the Aristotle's lantern retractor muscles of Lytechinus variegatus with (A) colorimetric determination of acetyl cholinesterase activity (static enzyme assay) and (B) cholinesterase staining. Experimental animals were maintained in their respective treatments for 4 weeks before experiments. ASW, artificial seawater; IP, inorganic (sodium) phosphate; OP, organic (triethyl) phosphate. n 5 5 individuals treatment –1; bars with different letters (a–d) are statistically significant from one another).

significantly lower levels (P 5 0.002) of AChE activity when compared with individuals maintained in seawater controls or inorganic phosphates. Decreasing levels of enzyme activity observed in muscles of individuals maintained in organic phosphate treatments were inversely correlated with increasing phosphate concentrations.

A similar pattern was observed when lantern retractor muscles of the Aristotle's lantern were stained to examine cholinesterase activity [Fig. 1(B)]. There were no significant differences (P 5 0.079) in the incidence of stained vesicles in individuals maintained in inorganic phosphate treatments compared to initial individuals or individuals maintained in the seawater control. In contrast, the number of stained vesicles decreased significantly (P 5 0.019) in individuals maintained in increasing organic phosphate concentrations once again, displaying an inverse dose response. Partially stained vesicles were observed in initially sacrificed individuals, individuals maintained in artificial seawater, the lowest concentrations of both phosphates, and the highest concentration of inorganic phosphate. There were no significant (P 5 0.12) differences in the incidence of partially stained vesicles among these treatments.

Maximum Force of Contraction

At a stimulation of 20 V, there was no significant (P 5 0.6) difference in the maximum force of contraction between the initially sacrificed individuals and individuals maintained in artificial seawater, low, or medium concentrations of inorganic or the low concentrations of organic phosphates (Fig. 2). Individuals maintained in the highest concentration of inorganic phosphate displayed a maximum F2

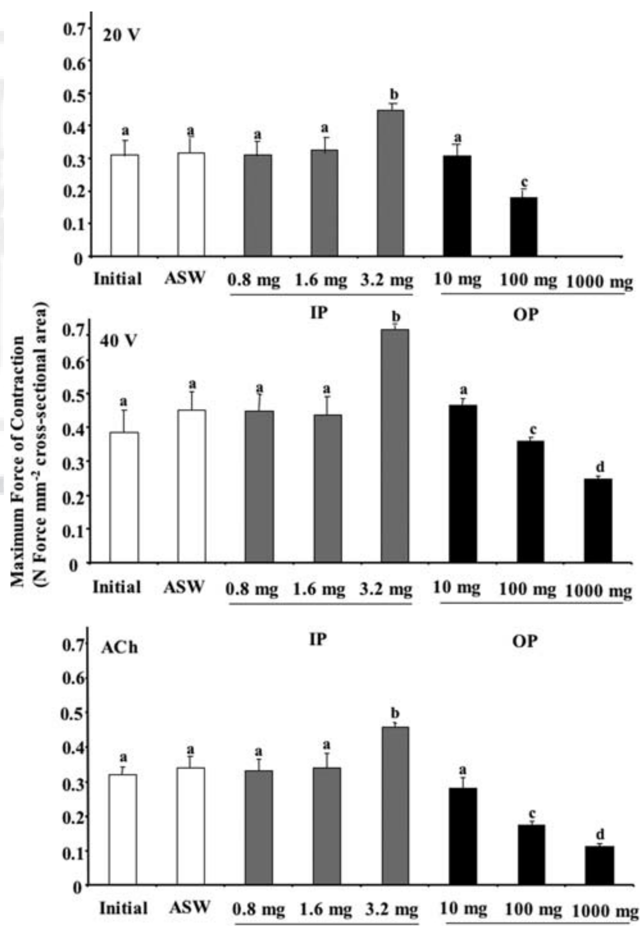

Fig. 2. Maximum force of contraction (tetanus) in the Aristotle's lantern retractor muscle of Lytechinus variegatus stimulated with 20 V, 2 ms, and 10 pulses/s; 40 V, 1 ms, 10 pulses/s, and 0.1 1M ACh. Experimental animals were maintained in their respective treatments for 4 weeks before experiments. ASW, artificial seawater; IP, inorganic (sodium) phosphate; OP, organic (triethyl) phosphate. n 5 5 individuals treatment –1; bars with different letters (a–d) are statistically significant from one another).

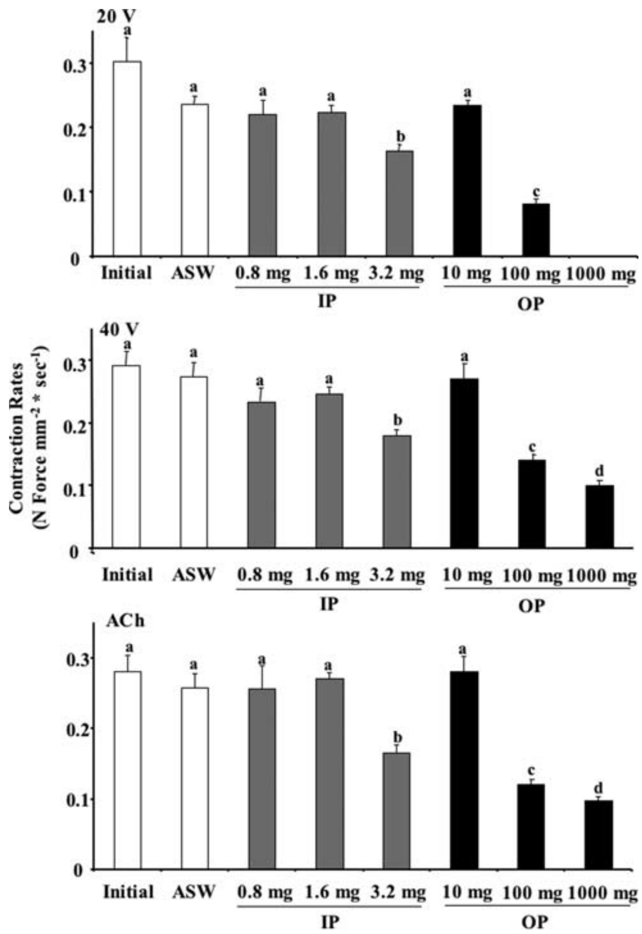

Fig. 3. Contraction rates in the Aristotle's lantern retractor muscles of Lytechinus variegates stimulated with 20 V, 2 ms, and 10 pulses/s; 40 V, 1 ms, 10 pulses/s, and 0.1 1M ACh. Experimental animals were maintained in their respective treatments for 4 weeks before experiments. ASW, artificial seawater; IP, inorganic (sodium) phosphate; OP, organic (triethyl) phosphate. n 5 5 individuals treatment –1; bars with different letters (a–d) are statistically significant from one another).

force of contraction significantly (P 5 0.043) higher than controls while values for individuals maintained in medium and high concentrations of organic phosphate decreased significantly (P ＼ 0.001) when compared with control treatments. Similar patterns were observed in muscles of individuals stimulated with 40 V and 0.1 1M Ach, whereas in individuals from all treatments showed maximum stimulation at 40 V when compared with stimulations with 20 V or 0.1 1M ACh.

Retractor Muscle Contraction Rates

Individuals in all treatments displayed increased rates of contraction when stimulated with 40 V compared to that of 20 V or 0.1 1M Ach (Fig. 3). There were no significant difference (P 5 0.6) in rates of muscular contraction in initially sacrificed individuals compared to individuals maintained in artificial seawater, low, or medium concentrations of inorganic phosphate or the lowest concentration of organic phosphate. Individuals maintained in the highest concentration of inorganic phosphate and the medium and high concentrations of organic phosphate displayed significantly (P ＼ 0.001) decreased rates of muscle contraction compared. An inverse dose response was observed in individuals maintained in organic phosphates under all three stimulation regimes (Fig. 3). F3

Retractor Muscle Relaxation Rates

Relaxation rates of the retractor muscle could be measured only for sea urchins receiving an electrical stimulus. When stimulated with 20 V, muscle relaxation rates of individuals maintained in artificial seawater and low and medium concentrations of inorganic phosphate did not differ significantly (P 5 0.46) from retractor muscle relaxation rates of initially sacrificed individuals (Fig. 4). Individuals maintained in the F4

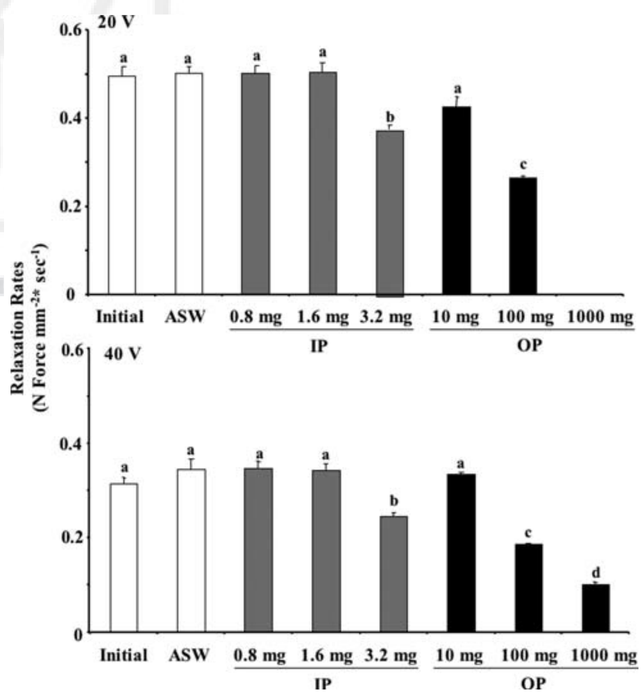

Fig. 4. Relaxation rates in the Aristotle's lantern retractor muscles of Lytechinus variegates stimulated with 20 V, 2 ms, and 10 pulses/s and 40 V, 1 ms, 10 pulses/s. No relaxation rates were measured for individuals stimulated with 0.1 1M ACh due to the problematic clearance of the chemical from the muscle. Experimental animals were maintained in their respective treatments for 4 weeks before experiments. ASW, artificial seawater; IP, inorganic (sodium) phosphate; OP, organic (triethyl) phosphate. n 5 5 individuals treatment –1; bars with different letters (a–d) are statistically significant from one another).

highest concentration of inorganic phosphate and the low and medium concentrations of organic phosphate displayed significantly (P 5 0.003) reduced muscle relaxation rates. All trends were similar in individuals stimulated with 40 V, with significantly lower (P 5 0.03) rates of muscle relaxation.

DISCUSSION

Reductions of AChE activity measured in the retractor muscles of the Aristotle's lantern of the sea urchin *Lytechinus variegatus* are similar to those measured in developing *Strongylocentrotus intermedius* and *S. nudus* treated with organophosphate pesticides (Turpaev and Semenova, 1988). Because *L. variegatus* were exposed chronically, in contrast to acute exposure in *Strongylocentrotus intermedius* and *S. nudus*, no recovery of AChE activity was measured, and muscular cholinesterase activity in *L. variegatus* exposed is likely to have been inhibited continuously for the duration of this experiment. The cholinergic system is responsible for muscle relaxation in echinoids (Binyon, 1972; Hill, 1993) with cholinesterase inhibition leading to overstimulation of muscles through acetyl choline, causing tetanus in muscles indicating that a tetanic state of the muscle system is likely to have occurred in *L. variegatus* maintained in the medium and highest concentration of organophosphates. These individuals displayed no spine movement or locomotion, were unable to extend their tube-feet, and possessed a rigid peristomial membrane that failed to exhibit the typical retraction response following stimulation with a sharp object (Böttger, personal observation). Our results suggest that, under chronic organophosphate exposure, triethyl phosphate binds to AChE evidenced by the lack of functional AChE. However, a reversal could take place after removing the stress of organophosphate exposure through biosynthesis of new AChE (Harris et al., 1971; Cisson and Wilson, 1977, 1981).

Echinoids are known to exhibit muscular contraction through single myosin control (Lehman and Szent-Györgyi, 1975; Hill, 1993). Calcium influxes into muscle cells trigger contractions by regulating protein ligation to myosin and enabling interaction between actin and myosin (Kendrick-Jones et al., 1970, 1972; Lehman et al., 1973; Lehman and Szent-Györgyi, 1975; Hill, 1993). This form of muscular control is found in smooth muscles of many invertebrates. Although structurally and functionally simpler than arthropods (Hoyle, 1983), echinoids possess several discreet muscles that exhibit higher degrees of development including those associated with the Aristotle's lantern (smooth muscle) and pedicellariae (striated muscle) (Hill, 1993). The force of muscle contraction in *Lytechinus variegatus* exposed to inorganic phosphates displayed similarities to muscular contractions in *Arbacia punctulata* exposed to identical phosphates (Böttger and Klinger, 1998). The linkage between the increase in maximum force of contraction and the increased levels of inorganic phosphate may be related to changes in the internal ionic distribution within the muscles of the Aristotle's lantern of *L. variegatus* (Hill, 1993). Because the influx of sodium and subsequent efflux of potassium is coupled with calcium release in echinoid muscles, which in terms triggers contraction (Lehman et al., 1973), an increase in sodium influx into the muscle may therefore be responsible for the increased force of contraction.

The dose-dependent decreases in maximum force of contraction observed in muscles of *Lytechinus variegates* in increasing concentrations of organic phosphate may be the result of the chronic nature of phosphate exposure, as muscles of echinoids experiencing acute organophosphate (anticholinesterase) exposure display enhanced contractions (Pentreath and Cobb, 1972). *L. variegatus* maintained in organophosphate concentrations for a month displayed decreased feeding rates and nutrient assimilation (Böttger et al., 2001), indicating that the changes in force of muscle contraction may be indirectly driven by starvation (Shizgal et al., 1986) rather than direct organophosphate induced changes in the muscle physiology.

Our experiments confirmed the presence of a specific cholinesterase (AChE) in the synaptic vessels of *Lytechinus variegatus* also observed in other echinoderms (Bacq, 1937; Augustinsson and Gustavson, 1949; Pentreath and Cottrell, 1968; Medes et al., 1970; Pentreath, 1970; Pentreath and Cobb, 1972). This enzyme is known to be responsible for inactivating liberated ACh through hydrolysis (Turpaev and Semenova, 1988). Acute exposure of sea urchin muscles to anticholinesterases stimulates contraction in echinoid muscles due to increased muscle stimulation through ACh (Pentreath and Cobb, 1972; Kobzar and Shelkovnikov, 1985; Turpaev and Semenova, 1988). Because AChE is important in muscle relaxation (Turpaev and Semenova, 1988), the sea urchin body will counteract the irreversible bond between AChE and the organophosphate by increased production and bioaccumulation of AChE (Harris et al., 1977; Cisson and Wilson, 1977, 1981; Turpaev and Semenova, 1988).

When organophosphate concentrations increased above 10 mg L^{21}, the overproduction of AChE is not sufficient to counteract the effects of the irreversible enzyme-organophosphate bonds as observed through the static enzyme assay and the staining of AChE containing vesicles. Organophosphate concentrations above 10 mg L^{21} triethyl phosphate lead to an AChE inhibition above 50% in *Lytechinus variegates*, which is the level at which signs of organophosphate poisoning are expressed in animals (Rand, 1995). Therefore, individuals maintained in the lowest organophosphate concentration would not be expected to display signs of organophosphate poisoning, such as impaired mobility. In fact, impaired mobility was expressed in individuals maintained in the medium and highest concentration of organic phosphate only (Böttger, personal observation), with the most pronounced effects in individuals maintained in the highest organic phosphate concentration. Any inhibition of AChE above 50% could therefore cause the muscle rigor observed, which we suggest could cause the decreased feeding (Böttger et al., 2001). Therefore, although changes in muscle force may be attributed to decreased feeding, this decrease could be directly related to AChE inhibition and subsequent muscle rigor.

In summary, organic phosphate-induced changes in muscular activity add yet another potential dimension to the overall compromised health of echinoids exposed to phosphate pollution. Previous experiments revealed that aspects of nutrition, reproduction, and righting behavior are also known to be compromised due to stress induced by exposure to inorganic and organic phosphates (Böttger and McClintock, 2001; Böttger et al., 2001). This study indicates that muscular function of *Lytechinus variegatus* occurring in drainage areas within the northern Gulf of Mexico containing organic phosphates is may be compromised. A tetanic muscular state is likely to inhibit normal locomotory and foraging behaviors with resultant negative effects on nutrition and reproduction. As *L. variegatus* plays an important ecological role in determining community structure in nearshore seagrass communities (Valentine and Heck, 1991; reviewed by Watts et al., 2001), changes in population demography, resulting from phosphate-induced physiological changes, may have community-wide ramifications.

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