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CHOLINESTERASE ACTIVITY AS A DEVICE FOR BIOMONITORING PESTICIDE EXPOSURE IN THE FRESHWATER MUSSEL *ELLIPTIO COMPLANATA*

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Cholinesterase Activity as a Device for Biomonitoring Pesticide Exposure in the Freshwater Mussel *Elliptio complanata*

Abstract: A die-off of freshwater mussels in 1990, attributed to anticholinesterase pesticide contamination of a North Carolina stream, has led the National Biological Service and the U. S. Fish and Wildlife Service to explore the development of biomonitoring programs using cholinesterase activity to assess the threat of anticholinesterase pesticides to freshwater mussels. However, background information such as "normal" cholinesterase activities and basic biochemical properties of the cholinesterases present in mussels is extremely limited. Early attempts to identify baseline cholinesterase activities for field-collected eastern elliptio (*Elliptio complanata*) have been plagued by high levels of variation in activities measured in mussels exposed to the same environmental conditions. The objectives of this study were two-fold: 1) to elucidate and reduce this variability through the characterization of the cholinesterases involved and the refinement of assay protocols, and 2) to continue for a second year the biomonitoring of cholinesterase activities in *E. complanata* in the area of the Tar River basin, North Carolina, where the die-off occurred. Enzyme characterization studies discovered that cholinesterase activities in crude homogenates of adductor muscle demonstrated inhibition with increasing substrate concentrations, preferred acetylthiocholine as a substrate over butyryl- and propionylthiocholine, and was not significantly inhibited by a specific butyrylcholinesterase inhibitor, suggesting that the predominately active enzyme in adductor muscle tissue is acetylcholinesterase. Minor improvements to the assay protocols did not lower the overall variation in activities of field samples. Coefficients of variation for each collection event still ranged from 24.12% to 65.46%. However, despite the large intra-site variation in ChE activities, the Hilliardston collection site, located near the 1990 die-off site, did reveal a significantly ($p < 0.05$) lower average cholinesterase activity (94.41 ± 46.05 μmoles substrate hydrolyzed/min/g protein) than the Berea reference site (141.20 ± 58.36 μmoles substrate/min/g protein). Evidence was insufficient to conclude that the decrease in cholinesterase activity at the die-off site was due to anticholinesterase agents. It is suggested that the variation in cholinesterase activities is mainly influenced by characteristics of the individual rather than measured water quality parameters. These characteristics are discussed along with recommendations for improving the biomonitoring program.

Key words: cholinesterase activity, anticholinesterase pesticides, acetylcholinesterase, assay refinement, freshwater mussel, *Elliptio complanata*, Tar River

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Questions, comments, and suggestions related to this report are encouraged. Inquires should be directed to the Service at the following address:

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Introduction

Although freshwater mussels of the families Margaritiferidae and Unionidae are presently found throughout the world, the continent of North America was at one point home to the greatest diversity of these mussels with 281 species and 16 subspecies native to the continent (Williams et al. 1993). However, mussel diversity has declined over the past 30 years. It is estimated that approximately 72% of North America's mussel species are now considered endangered, threatened, or of special concern (Williams et al. 1993). Of the freshwater mussel species inhabiting North Carolina, approximately 64% are also classified in these groups (Williams et al. 1993). The main causes of the decline are believed to be related to the destruction and alteration of mussel habitat. The construction of dams, other alterations in stream flow, and siltation due to erosion from adjacent deforested lands have all contributed to the demise of the North American mussel fauna. Although no longer a wide-spread practice, commercial harvest of some species also reduces population sizes, while particular collection practices destroy habitat. Finally, the introduction of competitively superior, exotic species, such as the zebra mussel (*Dreissena polymorpha*) and the Asiatic clam (*Corbicula fluminea*), has also, in effect, limited the habitat available to support large populations of native mussels. Although toxicants such as heavy metals, pesticides, and acid mine drainage have long been acknowledged as potential threats to many aquatic organisms, comparatively little work has been conducted on anticholinesterase pesticide impacts to freshwater mussels.

Anticholinesterase pesticides are primarily synthetic toxicants, more specifically known as organophosphorus and carbamate pesticides, which can either reversibly or irreversibly inhibit the hydrolytic activity of a group of synaptic enzymes collectively called cholinesterases (ChE's) (Abou-Donia 1995). The inhibition of ChE prevents the metabolic breakdown of choline neurotransmitters such as acetylcholine and prolongs signal transmissions through the synapse. Consequently, lethal paralysis may result through the over-stimulation of the nervous system. Anticholinesterase pesticides are introduced to the environment mainly through agricultural applications, and there have been several documented cases in which these pesticides have had deleterious effects on birds (White and Seginak 1990, Blus et al. 1991, Flickinger 1991, Wilson et al. 1991, Stansley 1993) and fish (Williams 1989, Stansley 1993). Anticholinesterase pesticides are usually rather water-soluble, so it is possible that streams running through or adjacent to agricultural areas could receive runoff which contained these pesticides, threatening the health of aquatic organisms such as mussels. Since eastern North Carolina is a largely agricultural area, anticholinesterase pesticide contamination of streams is a relevant concern, especially if the contamination could affect the health of federally listed endangered or candidate freshwater mussel species of the area. Because freshwater mussels depend heavily upon healthy host fish populations and good water quality for reproduction and survival, mussels are considered to be useful indicators of ecosystem health (Williams et al. 1993). Although many species of bivalves have been used as biomonitoring devices for heavy metals and organochlorine compounds (Doherty 1990, Mersch et al. 1992, Metcalfe-Smith 1994), very little research has been done on the effects of agricultural anticholinesterase runoff on freshwater mussels. Only recently has interest in this area developed, stemmed by reports of the first mass mortality of freshwater mussels theorized to be the result of exposure to anti-ChE pesticides.

In August 1990, a massive freshwater mussel die-off was discovered in Swift Creek, Nash County, North Carolina (Fleming et al. 1995). Approximately 10% of all mussels affected by the die-off (i.e. found dead or moribund) were individuals of the endangered Tar spinymussel (*Elliptio steinstansana*). Laboratory analysis of tissues taken from dead mussels indicated that the mortality was of an acute nature, giving suspicion as a possible cause to anticholinesterase agents which were known to be used in nearby agricultural

Methods

Freshwater mussels of the species *E. complanata* were collected from two North Carolina streams located within the Tar River system (Fig. 1). Collection sites were given names referring to nearby communities. The collection site nearest the 1990 die-off area was located in Nash County on the Swift Creek tributary to the Tar River and was referred to as Hilliardston. It received drainage from a sub-basin of the Tar River watershed covering approximately 133 square miles. Land use around Hilliardston consists of primarily forestry and agriculture (Fleming et al. 1995). Agricultural crops include sweet potatoes, cotton, tobacco, corn, cucumbers and soybeans (Hoeppner 1990). Lannate (a.i., methomyl) and Orthene (a.i., acephate) were the most commonly used pesticides in the Hilliardston area in 1990 (Hoeppner 1990). The only point-source discharges upstream of Hilliardston (but downstream of our reference site) were outfalls for treated domestic wastewater, which approximated 0.01% of the average daily flow of the stream (McGrath 1992). A few farms may extract water from the stream for irrigation (McGrath 1992). The reference site, referred to as Berea, is located in Granville County, approximately 50 miles west-northwest of Hilliardston and receives drainage from a sub-basin of about 21 square miles. Land uses in the Berea area are also predominately agriculture and forestry. Berea was chosen as the reference site because it was the most upstream, easily accessible location in the Tar River basin which supported a population of *E. complanata* large enough to fulfill sampling requirements without excessively depleting the population.

At each site, individual mussels with a shell length of ≥ 65 mm were extracted from the stream bed by hand and placed collectively in a plastic bag containing ice. Upon returning to the laboratory later the same day, the mussels were transferred to a -20°C freezer, in which they were stored until needed for assay. Ten individuals of *E. complanata* were collected per site per sampling day every 10-12 days from late June 1994 through late August 1994. This period of time bracketed the 1990 die-off event and was also the period during which a significant depression in AChE activity at Hilliardston was observed during 1993 Phase 1 monitoring. In addition, the application of Lannate and Orthene is prescribed mainly during the summer (Hoeppner 1990). The frequency of collections decreased to once every three weeks, five individuals from each site, after September 3, 1994, in order to spare the mussel communities from a possible decrease in reproductive success caused by depletion of the population, while still allowing seasonal variation in ChE activity to be monitored. Moreover, collecting at intervals of 21 days should have allowed the detection of unexpected exposures to some anticholinesterases, since studies showed that significant ChE depression resulting from sublethal exposure to 5 ppm acephate was still evident in *E. complanata* 24 days post-exposure (Moulton et al. 1995). Collection efforts were terminated in February 1995. Water quality data were collected on site during each collection event with a portable dissolved oxygen meter (YSI Model 51B, Yellow Springs Instrument Co. Yellow Springs, OH) and a portable conductivity meter (YSI Model 33 S.C.T. meter, Yellow Springs Instrument Co.).

Tissue preparation

Frozen mussels were pried open with an oyster knife. This procedure neatly separated the frozen visceral tissue and the posterior and anterior adductor muscles from one of the valves. The desired adductor muscle was then excised through this area of detachment, excluding the outer-most layers of the muscle in order to avoid contamination of the excised tissue with encroaching visceral tissue. For some studies, adductor muscles were arbitrarily but similarly divided into regions during dissection (Fig. 2). The excised tissue was then stored in plastic centrifuge tubes at -20°C until needed. The adductor muscles were chosen for assay because this tissue is easily

specificity, a 4.73 mM final concentration of butyrylthiocholine iodide (BuSChI) or propionylthiocholine iodide (PrSChI) was used in ChE activity assays in place of ASChI. The resulting enzyme activities were compared to those of equal concentrations of ASChI.

BuChE inhibition assay

In addition to the substrate inhibition and specificity assays, a BuChE inhibition assay was conducted in order to further characterize the cholinesterases present in the adductor muscle tissue. This assay examined the degree of inhibition in ChE activity which could be attained through the incorporation of a specific inhibitor for BuChE, tetraisopropyl pyrophosphoramide (isoOMPA) (Sigma Chemical Co., St. Louis, MO). A stock solution of 10^{-3} M isoOMPA was made using deionized water, from which dilutions of 10^{-4}, 10^{-5}, and 10^{-6} M were generated. Anterior adductor muscle tissue was prepared as described above and passed through glass wool. A 200 µl volume of homogenate was incubated on ice with an equal volume of inhibitor for 15 min. Then 2.8 ml of DTNB chromogen solution and 100 µl 0.156 M ASChI was added, and ChE activity was measured immediately at a reaction temperature of 25°C. Samples were analyzed in duplicate for each of the four inhibitor concentrations. The resulting ChE activities were compared to reference ChE activities obtained from homogenates which were not incubated in isoOMPA. Results are expressed as percent of reference activity.

Comparison of ChE activity in muscle regions

The possibility of a heterogeneous distribution of cholinesterases in adductor muscle was investigated, since procedures followed during Phase 1 called for only a portion of the adductor muscle to be excised for assay. The extraction of inconsistent portions of muscle may contribute to the variability in cholinesterase activity if cholinesterases are not distributed homogeneously throughout the muscle. The anterior and posterior adductor muscles from field samples collected from Berea in September 1994 was arbitrarily but consistently divided into regions and excised (Fig. 2). Again, the outer edges of the adductor muscle were avoided in order to prevent contamination of the excised tissue with intruding visceral tissue. Muscle regions were homogenized and were allowed to settle at 4°C for at least 2 hours. The clear liquid at the bottom of the tube and underneath the gelatinous foam was used for the enzyme activity assay. Anterior and posterior adductor muscles were analyzed separately since their ChE activities are known to differ (Moulton et al. 1995).

Enzyme stability

Phase 1 protocols called for allowing the sample homogenates to settle for 16-24 hours at 4°C. Since the reliability of enzyme assays depends upon the integrity of the enzyme, which is affected by temperature and duration of tissue storage, an assay was conducted to assess the stability of ChE under Phase 1 protocols. Posterior adductor muscle regions were homogenized and allowed to settle at 4°C as was done for the muscle region study. ChE activities were measured after 2, 8, 20.5, 32, 44, 56, and 80.5 hours of settling at 4°C.

Cholinesterase solubility

Tissues were homogenized in 25 volumes 0.5 M Tris buffer (pH 8 at 25°C) and allowed to settle at 4°C for approximately one hour. A 1 ml aliquot of the clear homogenate was then collected and passed through glass wool. ChE

in the anterior adductor muscle than in the posterior adductor muscle. Representing the same data as μmoles substrate/min/g wet weight increases the CV over that for protein-normalized activities.

Enzyme stability assay

In the enzyme stability assay, the CV's for ChE activities in muscle regions within an individual increased with time spent settling at 4°C after homogenization. After 2 hours of settling at 4°C, the ChE activities showed a coefficient of variation of 16.8%. This increased to 28.0% after 20.5 hours and to 43.7% after 80.5 hours (Fig. 5). This assay also reinforced the suspicion that ChE activity is not homogeneously distributed throughout the posterior adductor muscle. All muscle regions, with the exception of the comparison of Post 5 and Post 2, displayed statistically different average ChE activities over the 80.5 hours when compared to each other by single factor ANOVA ($\alpha = 0.05$).

Cholinesterase solubility

All homogenates treated with Triton X-100 yielded higher ChE activities than did non-treated homogenates (Table 2). Incorporation of detergent into the homogenization process produced an average increase in ChE activity of 235%. Samples treated with detergent also produced a lower coefficient of variation (24.86%) compared to samples not treated with detergent (32.53%).

Field samples

The Hilliardston site was characterized by significantly smaller individuals than the reference site, with average shell lengths of 75.18 ± 6.85 mm and 96.41 ± 12.11 mm, respectively. The Hilliardston site also had statistically lower average conductivity ($p < 0.05$) than Berea (Fig. 6). There was no significant difference in the average water temperature or dissolved oxygen content between the two sites (Fig. 7 and 8).

Overall, the Hilliardston site had a significantly lower average ChE activity (94.41 ± 46.05 μmoles substrate hydrolyzed/min/g protein) when compared to the reference site (141.20 ± 58.36 μmoles substrate/min/g protein) ($p < 0.05$) (Fig. 9). However, due to the large intra-site variability encountered during each sampling event, there were only five collection dates where a statistically lower average ChE activity ($\alpha = 0.05$) was apparent at Hilliardston. These dates included July 7 and 15, 1994, August 18, 1994, and November 5, 1994. There also appears to be a significant difference in ChE activities between sites during the January 20, 1995, collection event. However, mussels collected during the January sampling event were collected during high waters after a heavy rain. Consequently, only one individual was collected from the Berea site, and therefore, the apparent significant difference in ChE activity for that collection date may be misleading. Over the course of the study, coefficients of variation for ChE activities from individual sampling events ranged from 24.12% to 52.93% with an average of 38.33% (SD = 10.26%) for Berea and from 26.95% to 65.46% with an average of 44.08% (SD = 12.79%) for Hilliardston. ChE activities from an external standard of pooled adductor muscle tissue showed a coefficient of variation of approximately 9.15% (n=39) which represented the precision limit for the assay protocols.

The correlation coefficients for shell length, water temperature, conductivity, dissolved oxygen, and ChE activity are listed in Table 3. Although conductivity, water temperature, and dissolved oxygen were highly correlated to each other, separate regression analyses of the log of ChE

enzyme preferred ASChI over BuSChI and PrSChI as shown by the higher activities seen when ASChI was used as the substrate. Moreover, PrSChI was hydrolyzed at a faster rate than BuSChI. Augustinsson (1949) used these specific properties to distinguish acetylcholinesterase from other "choline ester-splitting enzymes." Therefore, the evidence suggests that the predominate cholinesterase in the adductor muscle crude homogenate is probably acetylcholinesterase. Inhibition studies using micromolar concentrations of isoOMPA, a specific inhibitor for BuChE, did demonstrate a 30% decrease in total ChE activity in the homogenate, signifying that AChE may not be the only esterase present in the tissue. BuChE is completely inhibited at concentrations on the order of 10^{-3} M isoOMPA (Abou-Donia, personal communication), so the persistence of most of the reference ChE activity at that concentration signifies the presence of AChE in the homogenate. However, one must use caution when relying upon a few characteristics to identify an enzyme. Most newly described enzymes are defined according to vertebrate characteristics, but these characteristics can often overlap when attempting to define invertebrate esterases (Oxford 1973, Habig et al. 1988, Ozretic and Krajnovic-Ozretic 1992). It should be kept in mind that the enzyme assays of this project were performed on crude homogenates, and so BuChE and PrChE may also be present in the tissues but with activities lower than AChE. Nevertheless, AChE seems to comprise the majority of the cholinesterase activity in the anterior adductor muscle, so the apparent mixture of enzymes should not be of consequence to the development of a biomonitoring project for anticholinesterase pesticides. Since the enzyme activities measured in field samples probably represented the activities of not only AChE, they are referred to in this report as simply ChE activities.

Cholinesterase solubility

ChE activities were increased by an average of 235% through the practice of homogenizing tissues in 1% Triton X-100. This was consistent with the results of Habig et al. (1988) in which channel catfish (*Ictalurus punctatus*) brain AChE activity was increased by 40-150%, depending upon the subcellular fraction assayed. Higher baseline ChE activities would make it easier to statistically identify a certain level of enzyme inhibition. In addition, mussel samples treated with detergent produced a lower coefficient of variation (CV = 24.86%) compared to those samples not treated with detergent (CV = 32.53%). This was perhaps the result of a more complete homogenization obtained by incorporating detergent into the sample. Despite these two beneficial results of solubilizing the proteins by incorporating Triton X-100 into sample homogenates, this practice was not performed on the field samples. No toxicological dose-response data were available for the effects of organophosphate pesticides on the detergent-soluble ChE activity in *E. complanata*. However, it has been demonstrated that certain organophosphate pesticides can inhibit the ChE activity in crude homogenates of *E. complanata* adductor muscle (Moulton and Fleming, unpublished data). Therefore, only crude homogenates were assayed for ChE activity in the field samples. However, Plummer et al. (1984) found that detergent-soluble AChE from various mammalian tissues can be more sensitive to inhibition by some organophosphorus pesticides (OPs) than the naturally soluble enzymes. In contrast, Habig et al. (1988) found that treatment of channel catfish brain and blue crab (*Callinectes sapidus*) ventral ganglion tissue with Triton X-100 in quantities sufficient to increase AChE activity did not also show an increase in the susceptibility to various OPs. Therefore, it would be beneficial to examine the toxicological effects of OPs on the detergent-soluble enzyme activity of *E. complanata* adductor muscle to see if indeed this form of the enzyme is more susceptible to OPs. If so, altering assay protocols to use the detergent-soluble enzyme activity may produce a more sensitive biomonitoring tool for organophosphorus pesticide exposure.

considered abnormally low, and after accounting for natural variations, (such as seasonal fluctuations, gender-biases, changes in metabolic status, etc.), can be considered to be due to exposure to anticholinesterase agents. Values characterizing baseline ChE activities have been published for a variety of bird species; these are often relied upon as reference values for investigations of avian poisonings. Because each case of animal exposure to anticholinesterase agents in the environment is a unique situation, the degree of ChE activity depression, or percent inhibition with respect to levels in concurrently collected reference specimens, is often recommended for purposes of comparison rather than comparison of ChE activity to pre-defined reference values (Hill and Fleming 1982). Regardless of whether pre-defined ChE values or ChE activities of concurrent reference specimens are the basis for a comparison, there is still a need for criteria on what level of inhibition is meaningful relative to anticholinesterase exposure and effect. For bird and fish brain ChE activities, a depression in live animals of 20% or more from average baseline activities is considered indicative of exposure to anticholinesterase agents (Hill and Fleming 1982). One of the criteria for diagnosing poisoning by anticholinesterase agents in dead birds is ChE inhibition of ≥ 50% (Hill and Fleming 1982). Moulton et al. (1995) offer the criterion of a 30% decrease in ChE activity as indicative of anticholinesterase exposure in freshwater mussels based on laboratory dose-response work.

Based on this study, the ability to define a baseline ChE value for *E. complanata* and the applicability of numerical criteria as to the significance of lowered ChE activities in mussels remains questionable. In Phase 2 studies, the ChE activities within a collection event at the reference site had CV's of 24% to 53%, meaning that ChE activities could be depressed by up to 53% in live mussels and still be considered "normal." High intrasite variability limits the discriminatory power of intersite comparisons. Mussels from Hilliardston had significantly lower ChE activities ($p < 0.05$) than those collected concurrently from Berea during only three sample events. On July 7, July 15 and August 17, average ChE levels in Hilliardston mussels were determined to be 50.9%, 51.4%, and 45.4% lower, respectively, than those from Berea specimens. During the mussel die-off in 1990, ChE activities in dead mussels were depressed only 65% relative to live mussels collected from the reference site. This suggests that the lethal threshold may occur within a narrow margin. A protective biomonitoring project should detect the occurrence of abnormally low ChE activities before the lethal degree of inhibition is attained. Obviously, if the causes of the intrasite variability can be elucidated, the range of CV's may be narrowed, making the criterion for indicating anticholinesterase agent exposure more easily and confidently defined. Nevertheless, the rough estimates of baseline ChE activities presented by this project can provide a guideline to assessing ChE activities in field samples.

As was discovered in the Phase 1 results, mussels from Hilliardston had significantly lower ChE activities ($p < 0.05$) than those collected concurrently from Berea during several sample event in summer. Although significant difference were observed between stations in 1994, there was no overall decreasing trend in ChE activities within sites during this period at either site (i.e. during Phase 2 studies), in contrast to the decreasing trend in ChE activities which was apparent at Hilliardston during July 1993. In fact, the difference in the Phase 2 ChE activities found between the two sites seemed to result from an increase in enzyme activities at Berea, not from a deviation from the average ChE activity at Hilliardston. Therefore, no pattern of ChE activity trends was consistent between Phase 1 and Phase 2 studies. The period of ChE activity depression seen in the Phase 1 study does not seem to be a recurring threat to the Hilliardston mussel population. However, ChE monitoring must continue for at least one more year in order to be certain that activities seen in either year were not anomalous.

the other because of differences in biochemical and metabolic characteristics. For instance, highly lipid-soluble organochlorine pesticides can produce a greater risk of toxicity in females because of the additional lipid material associated with reproductive processes (Ecobichon 1991). Metcalfe-Smith (1994) observed differences in heavy metal accumulation between male and female mussels, which may be associated the differences in tissue composition. Unfortunately, the meager existing literature on gender differences in ChE activities concern vertebrate organisms. In female HAN-Wister rats, baseline plasma BuChE activity was found to be 5.5 times greater than in male rats (Schmidt and Schmidt 1978). In Japanese quail, male birds exposed to carbaryl experienced greater plasma BuChE inhibition and slower recovery rates than female birds (Hill 1979). Hill (1989) also discovered that the stability of quail plasma BuChE activities during cold storage varied depending upon gender and previous inhibition by anticholinesterase agents. In rhesus monkeys, chronic exposure to anticholinesterase agents can have gender-biased effects mediated through a difference in de novo synthesis of plasma BuChE and erythrocyte AChE (Woodard et al. 1994). These studies, although focusing on vertebrates, suggest that gender can also influence ChE activities in invertebrate organisms. Gender may also indirectly influence ChE activities by affecting the induction of a chemical biotransformation system, known as the cytochrome P-450 monooxygenase enzymes, which is responsible for metabolizing and detoxifying anticholinesterase pesticides as well as many other xenobiotic compounds (Goksoyr and Forlin 1992). Unfortunately, the P-450 system has not been investigated in *E. complanata*, and again, the existing literature provides information for vertebrate animals. The P-450 system has been thoroughly studied in many species of fish, and it was found that some of these enzymes exhibit a sex-specific expression (Goksoyr and Forlin 1992). Pregnant rats were found to be more susceptible to paraoxon than virgin females because of a decrease in the ability to detoxify paraoxon, mediated through a reduction in serum paraoxonase activity (Weitman et al. 1983). Therefore, in addition to mere gender, reproductive status may also affect ChE activities.

The reproductive season starts in early May and can last into July. Therefore, the deviations from the average in ChE activities which we observed during the summer corresponds to the end of the reproductive season of *E. complanata*. During the reproductive season, female mussels encounter greater physiological demands in ensuring reproductive success than do males. A male releases sperm to the water column which fertilizes the ova within the female's suprabranchial chambers (Matteson 1948). Female mussels then nurture developing embryos, known as glochidia, for a period of about one month within the water canals of the outer gills, also known as the marsupia. Finally, mature glochidia are released into the water column via jets of exhalent water created by sudden contractions of the valves. During the time when glochidia are within the marsupia, the female may expend additional energy to aerate the obstructed canals of the outer gills. As a result of the differences in effort spent and stresses encountered during reproduction, a divergence in the normal ChE activities may be generated between males and females.

Unfortunately, determination of gender and reproductive status in mussels is difficult. *E. complanata* does not express sexual dimorphism (Matteson 1948). Therefore, the only way to determine the gender of an individual is to perform microscopic histological analysis of reproductive tissues (Matteson 1948). Individuals of *E. complanata* are usually dioecious, although the gonads are composed of varying combinations of male and female reproductive tissue. Approximately 80% of the individuals in a population of *E. complanata* located in Lac de l'Achigan, Quebec, were determined to be hermaphroditic to some degree (Downing et al. 1989). It was suggested that *E. complanata* reaches sexual maturity as a male and then develops into a female with age (Downing et al. 1989). If this is the case, perhaps the restriction of collecting mussels of a minimum size skewed the sample collected from the Berea site toward a predominance of female mussels. The determination of reproductive

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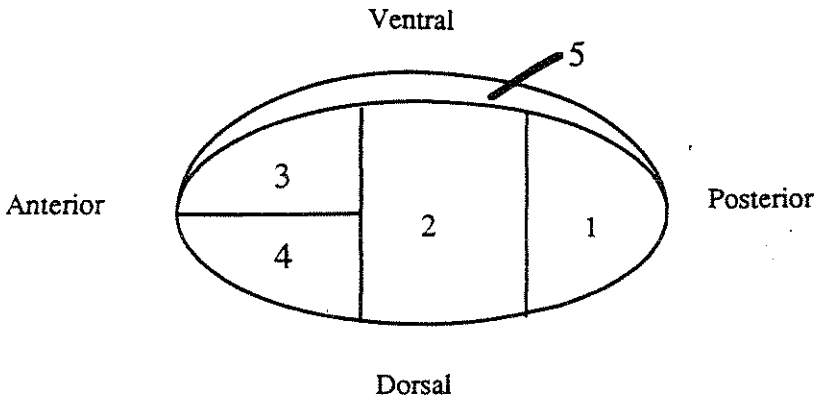

Fig 2: Diagram of arbitrarily designated muscle regions in posterior adductor muscle. For anterior adductor muscle, the region designated as Ant 1 is the anterior half of the muscle and Ant 2 is the posterior half.

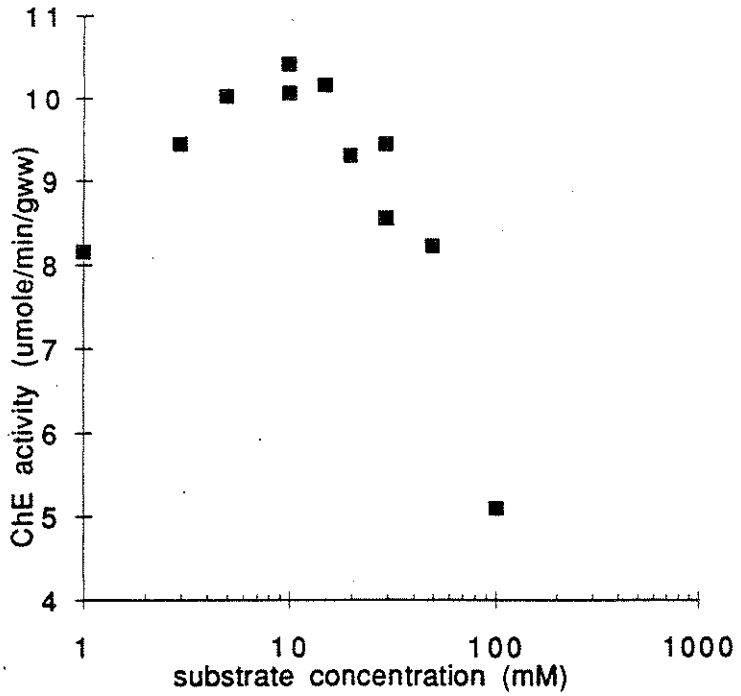

Fig. 3: Substrate inhibition assay using various concentrations of ASChI on homogenates of anterior adductor muscle.

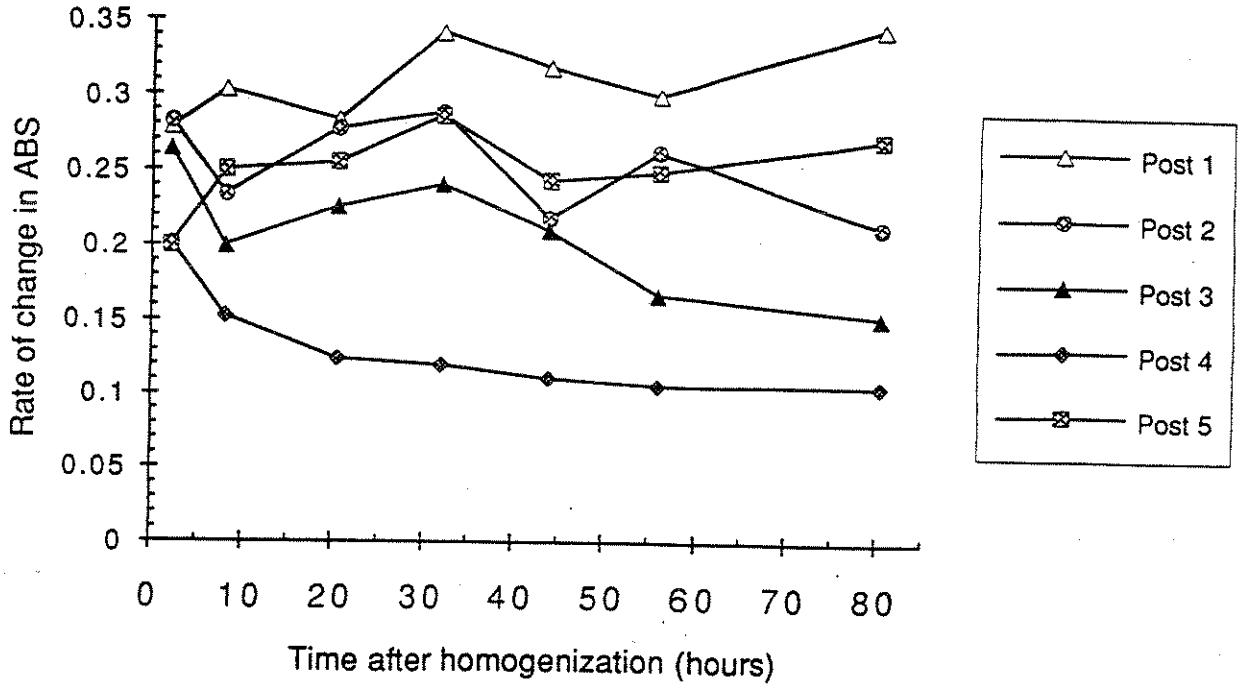

Fig. 5: Stability of ChE after homogenization and settling at various intervals at 4°C. Each data set represents a different muscle region. The rate of change in absorbance is directly proportional to ChE activity.

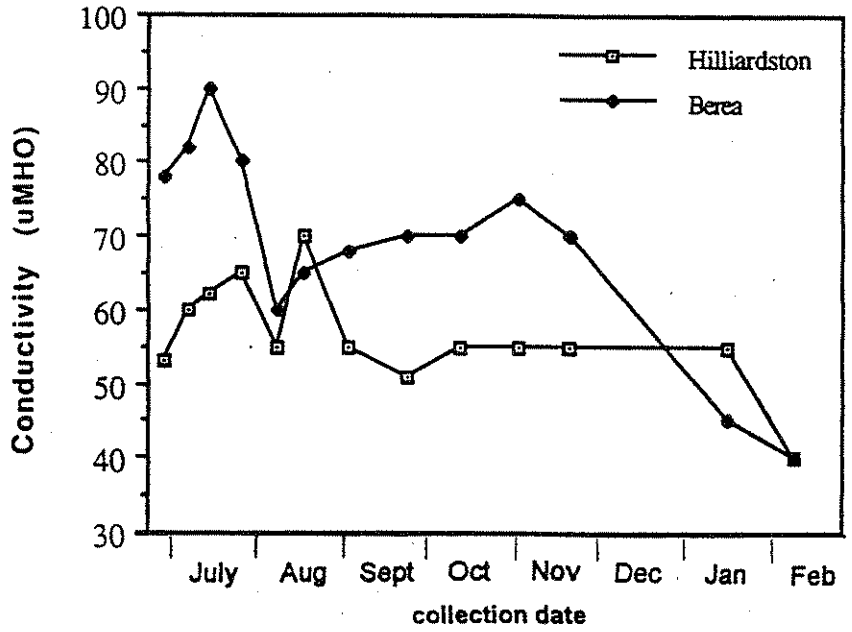

Fig. 6: Conductivity (μ MHO) of the stream water measured during each collection event.

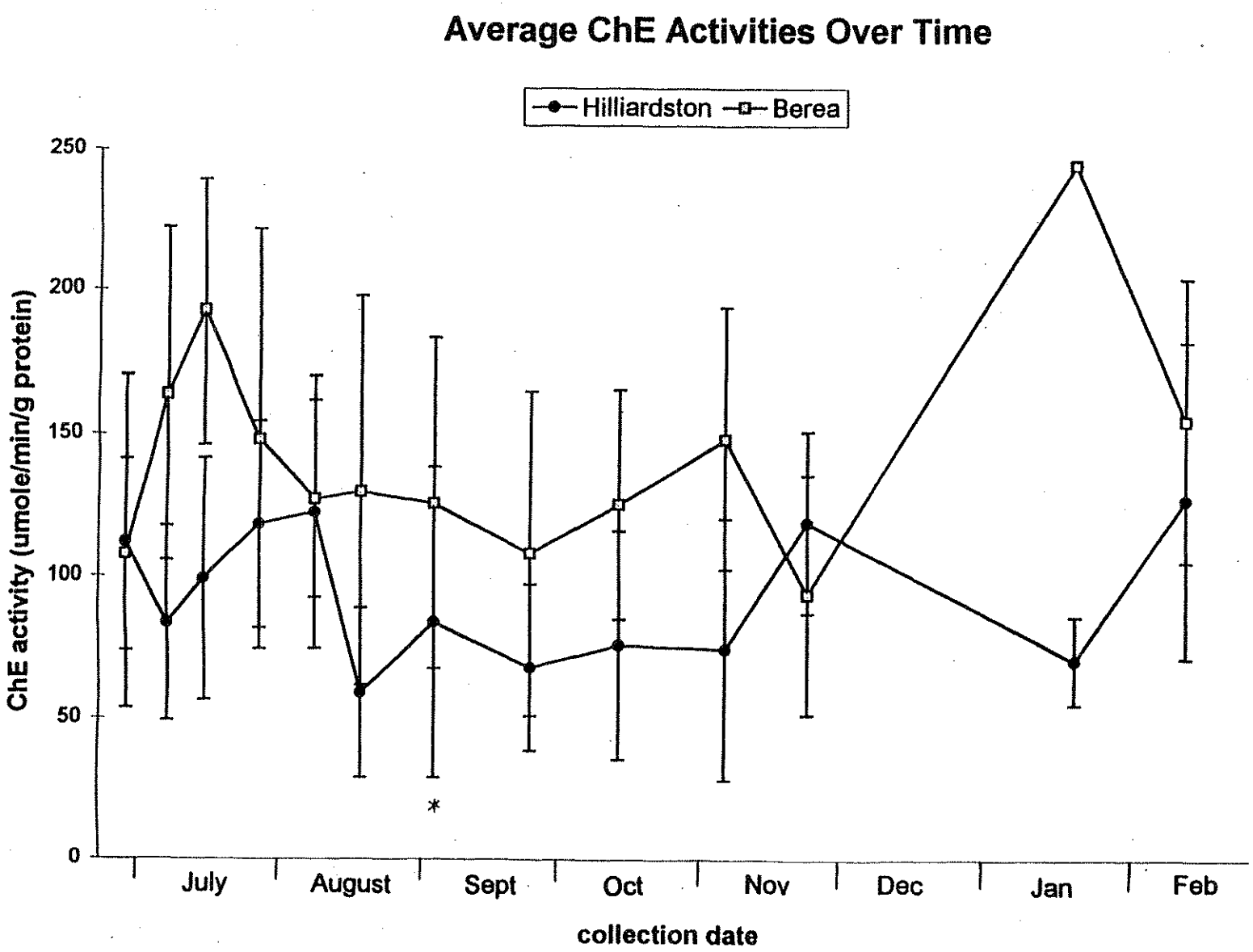

Fig. 9: Average ChE activities for the Hilliardston and Berea sites over time. For collection events through September 3, 1994, n = 10 per site per collection event. For collection events after September 3, 1994, (marked by an asterisk) n = 5. However, only one individual was collected from Berea on January 20, 1995. Error bars represent one standard deviation.

Hilliardston	shell length	water temperature	conductivity	dissolved oxygen	ChE activity
shell length	1.0000*				
water temperature	-0.2299*	1.0000*			
conductivity	-0.2168*	0.6961*	1.0000*		
dissolved oxygen	0.2704*	-0.8888*	-0.5070*	1.0000*	
ChE activity	-0.3464*	-0.0317	-0.1722	-0.1078	1.0000*

Berea	shell length	water temperature	conductivity	dissolved oxygen	ChE activity
shell length	1.0000*				
water temperature	0.1518	1.0000*			
conductivity	0.0701	0.7042*	1.0000*		
dissolved oxygen	-0.2450*	-0.6095*	-0.4303*	1.0000*	
ChE activity	-0.4079*	0.1419	0.1666	-0.0548	1.0000*

Table 3: Pearson product moment correlation coefficients for regression variables. Values marked with asterisks are statistically significant at $p \leq 0.05$. Units for each variable are as follows: shell length (mm), water temperature (degrees Celsius), conductivity (uMHO), dissolved oxygen (ppm), and ChE activity (umole substrate hydrolyzed/min/g protein).