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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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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

areas. Subsequent examination of cholinesterase activities in various mussel species collected from the die-off site revealed depressed enzyme activities relative to the activities in mussels from an upstream reference site. No other signs of physiological stress or disease were found. As a result of this event, the National Biological Service (NBS) and the U.S. Fish and Wildlife Service (FWS) sought to establish a biomonitoring project to assess the frequency and severity of the threat of anticholinesterase pesticides to the endangered freshwater mussels as well as to other mussel species. The research presented in this paper was done to support the implementation of such a biomonitoring project.

The FWS began investigating the use of cholinesterase activity as a biomonitoring tool, following protocols and recommendations of NBS researchers, in the spring of 1993 (Moulton et al. 1995). The 1993 phase (Phase 1) of the project consisted of monitoring acetylcholinesterase (AChE) activities in individuals of a common mussel, the eastern elliptio (*Elliptio complanata*), a species that was also affected by the die-off in 1990 and that could act as a surrogate for the endangered species. The AChE assays were conducted spectrophotometrically according to a technique modified from Hill and Fleming (1982). Results indicated that during the mid-summer, ChE activities in mussels from near the die-off site were depressed an average of 78% with respect to the reference site (Augspurger, unpublished data). Compared to mussels collected near the die-off site in the previous spring, ChE activities in live mussels from the same site in mid-summer were depressed by up to 83%. However, the average coefficients of variation (CV's) for the 15 sample sets ranged from 39 to 49%, making it difficult to define a statistically valid delineation separating normal ChE activities from depressed activities. Therefore, the second year of the project (Phase 2) was dedicated to further investigate the use of cholinesterase activities in *E. complanata* as a biomonitoring tool.

The ultimate goal of the Phase 2 studies was to define the normal baseline cholinesterase activities of *E. complanata* to which activities from mussels in threatened sites could be compared. This required elucidation of the cause of the high intra-site variability in ChE activities seen in previous samples. The objective was to at least understand the ramifications of these causes if eliminating them was not possible. The Phase 1 assays revealed that there was no consistent correlation between ChE activity and routinely monitored water quality parameters such as water temperature, dissolved oxygen, conductivity, and pH (T. Augspurger, unpublished data). Therefore, the possible causes explored in the second phase of the biomonitoring project include various aspects of preliminary assay protocols, much of which stem from the lack of knowledge concerning the type of cholinesterase present in *E. complanata*. Topics investigated include the identification of the type of ChE present, distribution of ChE in muscle, solubility of the enzyme, and the stability of the enzyme after homogenization. Once refinement of the assay protocol had been completed, cholinesterase activities in field samples were to be analyzed in order to establish baseline data and to determine if the depression in enzyme activity found during Phase 1 studies was a repeating seasonal occurrence. Any periods of abnormally low ChE activity may signify either exposure to anticholinesterase agents or, if such ChE depression is a normal seasonal occurrence, simply a period when mussel ChE may be particularly susceptible to the deleterious effects of additional ChE depression caused by anticholinesterase pesticide exposure. Because of their close phylogenetic relationship, the risk to *E. complanata* can be assumed to also be a risk to the endangered *E. steinstansana*. Such a risk may warrant special steps for protection of the endangered species, such as stricter regulation of pesticide use and enforcement of runoff buffer zones.

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

identified and extracted from the animal, unlike mussel neural tissue, and has consistently detectable levels of ChE activity (Moulton et al. 1995). Muscle tissue was homogenized in 1:25 w/v 0.5 M Tris buffer (pH 8 at 25°C) for 1 min using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY) with a Kinematica Power Control Unit at a setting of 5. Samples were kept on ice during the homogenization process. Homogenization transformed the sample into an opaque gelatinous foam which would not liquefy upon mild centrifugation and would interfere with the spectrophotometric analysis. Therefore, in order to acquire a transparent liquid, the homogenates were either allowed to settle at 4°C for up to 24 hr or were passed through a glass pipette containing a 5 mm plug of glass wool.

An external standard was prepared from pooled anterior and posterior adductor muscles from six mussels from the reference site. Tissues were homogenized as described above and allowed to settle overnight at 4°C. The transparent portion of the homogenate was divided into 1 ml aliquots and stored at -80°C until needed for assay.

ChE activity assay

The colorimetric assay for the determination of ChE activity was adapted from the modified Ellman technique described by Hill and Fleming (1982). A 200 µl volume of homogenate was added to a polystyrene cuvette containing 3.0 ml of 250 µM dithio-bis-nitrobenzoic acid (DTNB) chromogen solution (Sigma Chemical Co., St. Louis, MO). The chromogen had been previously warmed to 27°C using a water bath so that the final enzymatic reaction could occur at 25°C. A 100 µl volume of 0.156 M acetylthiocholine iodide (ASChI) solution (Sigma Chemical Co.) was added to the cuvette. The reaction mixture was stirred, and the rate of color change was monitored at 405 nm for 3 min sing a Shimadzu UV-260 spectrophotometer (Kyoto, Japan). Each sample was measured in duplicate. Protein content was determined using the bicinchoninic acid (BCA) protein assay.

Protocol refinement

Sample preparation and assay protocols were manipulated in an attempt to reduce the high intra-site variability in ChE activities observed during Phase 1 studies. Aspects investigated included the specific identity of the cholinesterase present in the adductor muscle tissue, the possibility of a heterogeneous distribution of cholinesterase in the adductor muscle, the stability of the enzyme after homogenization, and the solubility of the mussel cholinesterase during homogenization. All of these Phase 2 studies were carried out using adductor muscle tissue from mussels collected from the reference site.

Substrate Inhibition and Specificity Assay

The preferred substrate for AChE is the neurotransmitter acetylcholine (ACh). To determine if AChE is the specific type of cholinesterase present in the adductor muscle of *E. complanata*, a unique characteristic of AChE was exploited. AChE activity is inhibited at excessive substrate concentrations, so a substrate inhibition assay was performed using acetylthiocholine as the substrate. Anterior adductor muscle was homogenized as described earlier and passed through glass wool. A stock solution of 0.156 M ASChI was made, from which varying quantities were added to 200 µl of homogenate and DTNB chromogen. The volume of DTNB chromogen solution was adjusted depending upon the volume of substrate used, so that the final volume in the cuvette was always 3.3 ml. Final substrate concentrations ranged from 0.473 mM to 100 mM. Enzyme activities were measured as described above. To assess the substrate

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

activity assays were performed on this aliquot, representing ChE activity in crude homogenate. To the original homogenate, the appropriate amount of Triton X-100 (Sigma Chemical Co.) was added to the homogenates to make a 1% detergent concentration. The homogenate and detergent were mixed for 10 seconds and allowed to settle for one hour at 4°C. A 1 ml aliquot of the clear liquid was then collected, passed through glass wool, and assayed for ChE activity, representing ChE activity influenced by detergent solubilization.

Field Samples

ChE activities in field samples were measured in anterior adductor muscle. The entire adductor muscle was excised, excluding the outer edges of the muscle, and homogenized as described above, under *Tissue preparation*. Aliquots of the homogenates were then deposited in glass pipettes which contained a 4-5 mm plug of glass wool. A rubber pipette bulb was used to apply a slight pressure which pushed the homogenate through the glass wool, effectively re-liquefying the homogenate. ChE activities were measured immediately at a reaction temperature of 25°C. Protein content was determined using the BCA assay. Differences in ChE activities between collection sites and collection events were determined using single factor ANOVA. Relationships between ChE activity, various water quality parameters, and shell length were analyzed using simple and multiple regression.

Results

Protocol refinement

Substrate Inhibition and Specificity Assays

Maximum ChE activity in anterior adductor muscle was achieved at concentration of approximately 10 mM acetylthiocholine (Fig. 3), beyond which higher concentrations produced a decrease in enzyme activity. Calculated values of Km for ASChI as a substrate ranged 1.55×10^{-4} to 2.89×10^{-4} M. At substrate concentrations of 4.73 mM, PrSChI produced an activity approximately 3 times lower than that of ASChI, while BuSChI produced an activity approximately 7 times lower than that of ASChI.

BuChE inhibition assay

Incubation of the anterior adductor muscle homogenate with an equal volume of 10^{-6} M isoOMPA inhibited ChE activities to 70%, on average, of reference ChE activities (Fig. 4). Increasing inhibitor concentrations to 10^{-3} M further decreased ChE activity to as little as 50% of reference activities in two of the three tissue samples examined.

Comparison of ChE activity in muscle regions

Within a single individual, the ChE activities in the arbitrarily designated posterior muscle regions differed with an average coefficient of variation of 18.21% (Table 1). There was no apparent pattern in the distribution of ChE activity throughout these muscle regions. In other words, no one region consistently displayed the greatest nor the lowest ChE activity between individuals. In addition, the ChE activities, represented as μmoles substrate/min/g protein, found in the arbitrary muscle regions of anterior adductor muscle seem to have generally lower coefficients of variation (1.04 - 6.21%) than enzyme activities determined in posterior adductor muscle (16.59 - 19.82%). This suggests that the ChE activity may be more evenly distributed

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

activity on these variables showed that these parameters did not have a significant predictive value to ChE activity at either collection site. On the other hand, shell length did have a statistically significant relationship to ChE activity at each of the two sites. An increase in shell length slightly decreased ChE activity. However, shell length only explained about 13% of the variability in log(ChE activity) at Hilliardston and 16% of the variability in log(ChE activity) at Berea. The multiple regression of log(ChE activity) on shell length and conductivity for the Hilliardston site further increased the amount of variability which could be explained; adding conductivity to the multiple regression model explained an additional 7% of the variability in log(ChE activity). The best regression model for Hilliardston was log(ChE activity) = -0.0147 (shell length) - 0.0096 (conductivity) + 3.5780, with ChE activity represented as μmoles substrate/min/g protein (r^2=0.199, F=11.93, p<0.001, df= 2, 96). No improvements could be acquired in the regression model for the Berea site. The best regression model for Berea was log(ChE activity) = -0.0063 (shell length) + 2.7168, with ChE activity represented as μmoles substrate/min/g protein (r^2=0.158, F=16.5, p<0.001, df= 1, 88).

Discussion

Characterization of the cholinesterase enzyme

Although AChE has been measured in tissues of the marine blue mussel (*Mytilus edulis*) (Bocquene et al. 1990) and the freshwater mussel *Anadonta cygnea* (Salanki et al. 1967), it was uncertain whether this enzyme is present in significant amounts in the adductor muscles of *E. complanata*. Therefore, the initial use of the Ellman technique to measure AChE activity in *E. complanata* was presumptive but not without merit since some cholinesterases are known to be rather nonspecific in the substrates which can be hydrolyzed (Abou-Donia 1995). For instance, propionlycholinesterase can hydrolyze ACh but at a slower rate than it can hydrolyze propionylcholine. However, the Ellman technique is most valuable when the appropriate substrate for the enzyme present is used. When using only ASChI as the substrate in the enzyme assay of field samples, the assay is limited to detecting the activity of primarily AChE. Acetylcholinesterase is one of the most common cholinesterases and has a distinguishing characteristic in that high concentrations of substrate inhibit the enzyme's activity. If indeed AChE is present in the tissues of *E. complanata*, no data exist concerning the sensitivity of the mussel AChE to inhibition by excess acetylthiocholine, the substrate analog used in the assay. Other cholinesterases measured in the tissues of *M. edulis* included butyrylcholinesterase and propionylcholinesterase (Bocquene et al. 1990); these enzymes may also be present in *E. complanata*. It is therefore necessary that the types of cholinesterase present in *E. complanata* adductor muscle are identified, so that the assay can be optimized by providing the preferred substrate to the predominant enzyme. Ensuring that assay substrate concentrations are not exceeding the threshold would prevent assay results from being tainted with artificially depressed enzyme activities.

Preliminary steps have been taken within this project to precisely identify the type of cholinesterase present within *E. complanata* adductor muscle. Substrate specificity and inhibition assays demonstrated that the predominantly acting enzyme in the crude muscle homogenate was inhibited at ASChI concentrations greater than 10 mM. This is slightly lower than for bovine erythrocyte ChE which peaked in activity at slightly more than 10 mM substrate (Ellman 1961). The peak mussel ChE activity was also lower than the activity in catfish brain AChE and crab ganglia AChE which began to become inhibited at 5 x 10^{-3} M (Habig et al. 1988). The ChE assays on the field samples were conducted using a final ASChI concentration of 4.73 mM, so artificial inhibition through excess substrate concentrations should not have been encountered in field sample results. The predominate adductor muscle

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.

Protocol Refinement

High intra-site variability in ChE activities hindered the analysis of Phase 1 data, since no clear threshold was apparent between normal activities and enzyme activities that were depressed. The results of the assay refinement studies suggest that minor changes in assay protocols may lower coefficients of variation so that data analysis may be facilitated. First, the entire adductor muscle, excluding the outer-most edges, should be excised and homogenized for assay. Studies comparing the ChE activities of arbitrarily divided muscle regions within an individual posterior adductor muscle showed that the activities varied an average of 18.21%. This suggests that ChE activity may not be uniformly distributed throughout the posterior adductor muscle. Performing the ChE activity assay on an arbitrary portion of the muscle may introduce additional variation into the results when comparing enzyme activities between mussel individuals. In addition, although anterior adductor muscle showed a small difference in ChE activities between muscle regions, this tissue revealed a low average CV of 3.63%. Therefore, it is also recommended that ChE assays be conducted preferably on anterior adductor muscle rather than posterior adductor muscle.

Secondly, enzyme activity assays should be conducted as soon as possible after homogenization. The variation in ChE activities can increase the longer the samples remain at temperatures above freezing, as was shown in the enzyme stability studies. Allowing the muscle region homogenates to settle overnight at 4°C produced an unacceptable increase in the variation of the results. This could have been due to degradation of the enzyme, interactions of the enzyme with other intracellular components which affect the enzymes ability to hydrolyze ASChI, or if anticholinesterase pesticides are involved, spontaneous reactivation of the enzyme. Wachtendonk and Neef (1979) observed that cold storage of AChE from the hemolymph of *M. edulis* simply delays the degradation of the enzyme and cannot prevent it. For instance, 50% of the original activity of the AChE was lost after storage at -28°C for 80 days. Zinkl et al. (1987) observed a significant change in brain ChE activity of rainbow trout (*Salmo gairdneri*) after seven days of storage at 4°C. In order that samples are not required to settle at 4°C for an extended period of time, and so they can be assayed as soon as possible after homogenization, it is recommended that glass wool be used to liquefy the homogenates and remove any particulate matter which may interfere with spectrophotometric analysis.

Field samples

Examination of field samples revealed that there was a significant difference ($p < 0.05$) in the average ChE activities between the Hilliardston and Berea sites; the ChE activities at the Hilliardston site (94.41 ± 46.05 µmoles substrate hydrolyzed/min/g protein) were generally lower than at the reference site (141.20 ± 58.36 µmoles substrate/min/g protein). This finding was consistent with Phase 1 results. However, since the actual concentrations of any anti-ChE pesticides in the two streams were not known during either Phase 1 or Phase 2 studies, it cannot be concluded that the lower ChE activities seen at Hilliardston were the result of inhibition by pesticides alone or even in concert with other factors (other factors which may influence ChE activity will be discussed later in this section). Nevertheless, the average ChE activities for a particular site can be considered rough baseline ChE activities, which appear to depend upon the collection site. However, with such a high degree of variability in ChE activities within a collection site and date, it remains difficult to define a criterion for identifying contaminant-inhibited ChE activities in field samples of freshwater mussels.

Ideally, such a criterion should define the lowest ChE activity occurring normally, without the influence of contaminants, and accounting for natural variations in enzyme activity. ChE activities below the criterion can then be

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 $\geq$ 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.

Despite the minor improvements to the assay protocols, the average overall CV's for ChE activities within a sampling event were nearly the same for both Phase 1 and Phase 2 studies (39% and 38% for Berea, respectively, and 49% and 44% for Hilliardston). However, the protocol alterations did succeed in reducing the overall range of CV's from 9 - 87% to 24 - 53% at Berea and from 24 - 78% to 22 - 65% at Hilliardston. Very little of the variation in ChE activity could be explained through single and multiple regressions of log(ChE activity) on water temperature, conductivity, dissolved oxygen, and shell length. The weakness of these relationships suggests that the variation in ChE activity is more likely affected by characteristics of the individual mussels and not ambient water quality parameters. Although shell length explained less than 20% of the variability in regression analyses, shell length did have a statistically significant relationship to ChE activity. Age can be correlated to shell length (Downing et al. 1989, Matteson 1948), and so one can conclude that ChE activity may be slightly affected by age. However, because of the significant differences we documented in shell sizes between the two sites, the differences in ChE activities between the two sites may actually be underestimated. ChE activity slightly decreased with increases in shell length (13 to 16%). Therefore, if mussels collected from Hilliardston had been larger (i.e., comparable in size to those collected from Berea), even lower ChE activity measurements for Hilliardston mussels could have resulted. Therefore, collecting similar-sized mussels from both sites in the future may allow for a more truthful comparison of ChE activities. Additional factors which could affect ChE activity include pesticide exposure prior to collection, metabolic status, gender, and reproductive status. Unfortunately, no such information was available for Phase 2 data analysis.

One of the disadvantages of field monitoring is that collection sites are not controlled experimental environments, and many unknown influences can affect the results of field assays. Therefore, the specific quantification of enzyme activities presented in this study provided limited but useful information, since no information was available as to pesticide exposure prior to collection. Not only does the bioavailability of a pesticide influence the degree of exposure for a mussel, but the mussel has the ability to temporarily isolate itself from ambient water quality conditions by simply closing its valves and suspending ventilation. Although the majority of mussels collected were located by searching for ventilating individuals (since a gaping shell is easily distinguished from a rock underwater), it was impossible to know for what length of time the mussel had been ventilating prior to collection or for what duration was its last period of dormancy. In other words, the potential for pesticide exposure and the metabolic status of the mussels were unknown. Thus, ChE activities determined within a site could be skewed depending upon whether or not individuals were ventilating when a pulse of toxicant flowed through the area. A possible means of addressing this uncertainty is to conduct reactivation assays in which a "reactivator" such as pyridine 2-aldoxime methiodide (2-PAM) is introduced into the homogenate after ChE activity has been determined. The reactivator will dissociate most OPs from the ChE so that an uninhibited enzyme activity can be determined. If a significantly higher activity is seen in the reactivation assay than before the reactivator was introduced, then the mussels were being exposed to OPs before collection. Reactivation assays could also dispel the possible dependence of ChE activity on location. For these reasons, it would be beneficial to develop the protocols for reactivation assays in *E. complanata*. Since the length of time passed after ChE inhibition by anticholinesterase pesticides affects the productivity of reactivation assays (Stansley 1993), controlled laboratory studies, where mussels are exposed to known concentrations of anticholinesterase agents and then treated with 2-PAM, may be the best approach to investigate the application of reactivation assays on mussel adductor muscle tissue.

Other factors which could affect ChE activity in field samples are gender and reproductive status. In many cases, toxicants can affect one sex more than

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

status (i.e. number of ova and number of glochidia brooded in marsupia) likewise requires microscopic examination of tissues. In addition, special precautions should be taken when collecting female mussels, since *E. complanata* easily expel their young from the marsupia when disturbed or handled roughly (Matteson, 1948). Because such precautions were not taken by investigators while collecting mussels during the summer reproductive season, the effect of reproductive status on cholinesterase activity could not be determined for Phase 2 data.

In conclusion, there are many aspects of the individual mussel which may influence ChE activity. However, even if these unknowns are elucidated, there remains the possibility that the variation seen in both Phase 1 and Phase 2 intra-site ChE activities cannot be rectified. Day and Scott (1990) experienced coefficients of variation for AChE activities in various arthropods which ranged 34.6 to 55.4%, approximately the same range encountered in mussel ChE activities, when using the traditional spectrophotometer method. However, they found that a modification of the Ellman procedure for microplate assay produced considerably smaller coefficients of variation (9.3 - 25.5%) than the standard spectrophotometer technique. Therefore, perhaps refinement of the ChE assay for use with *E. complanata* merely requires a change in analytical equipment. Nevertheless, elucidating the influences of pesticide exposure prior to collection, metabolic status, gender, and reproductive status on the ChE activity of *E. complanata*, as well as further investigating the biochemical characteristics of the cholinesterases involved, are still important to understanding the physiology of all mussel species and are essential to developing a thorough biomonitoring program for the protection of these species.

Summary of recommendations for protocol refinement

Collect mussels of similar size from each study and reference site in order to prevent bias caused by differences in age.

Excise the entire adductor muscle (excluding the outer-most layer) for homogenization and ChE activity assay to eliminate complications from the possible non-homogeneous distribution of ChE throughout the muscle.

Use anterior adductor muscle for ChE activity assays, because anterior adductor muscle may have a more homogeneous distribution of ChE throughout the tissue than posterior muscle.

Assay ChE activities in muscle tissue as soon as possible after homogenization. By filtering the homogenate with glass wool, long periods of homogenate settling can be avoided.

Research on detergent-soluble ChE activity and its susceptibility to anticholinesterase agents is needed and may yield a more sensitive indicator for anticholinesterase agent exposure.

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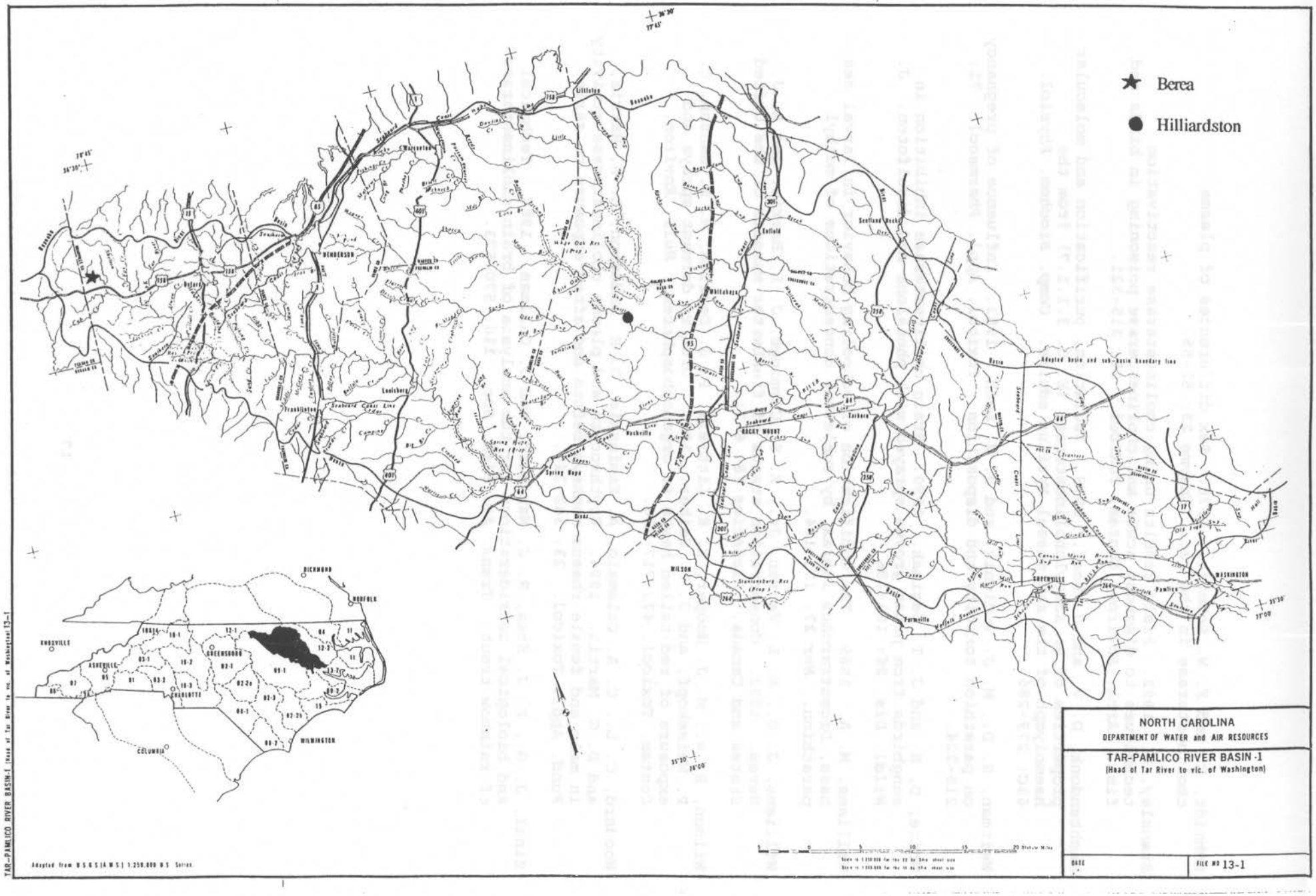

Fig. 1: Map of collection sites

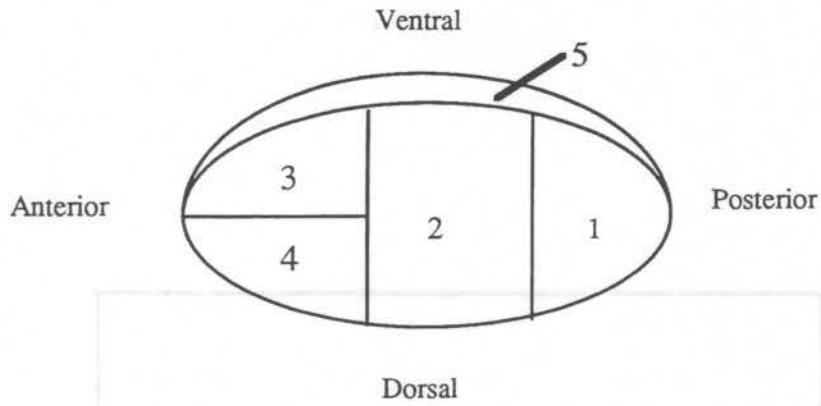

Fig 2: Diagram of arbitrarily designated muscle regions in posterior adductor muscle. For anterior adductor muscle, the region designated as Ant 1 is the anterior half of the muscle and Ant 2 is the posterior half.

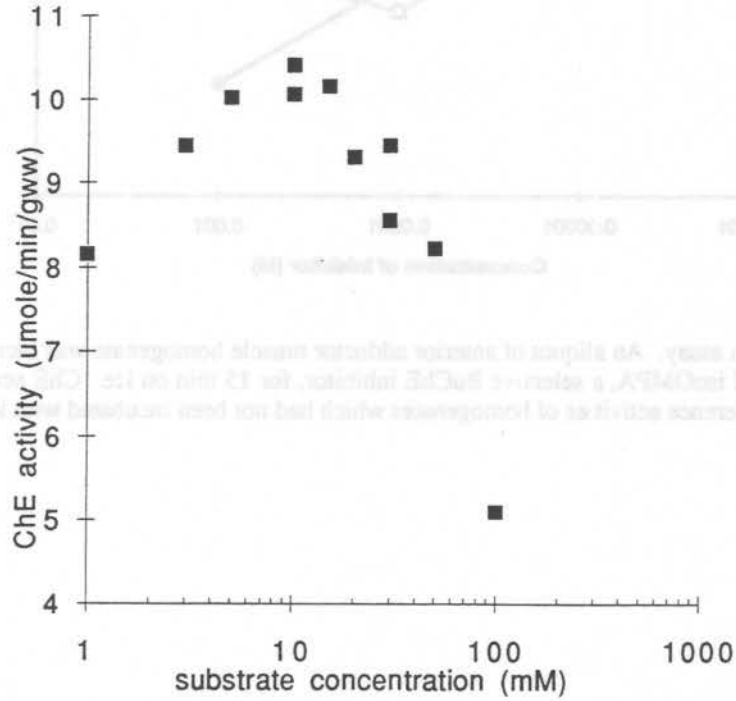

Fig. 3: Substrate inhibition assay using various concentrations of ASChI on homogenates of anterior adductor muscle.

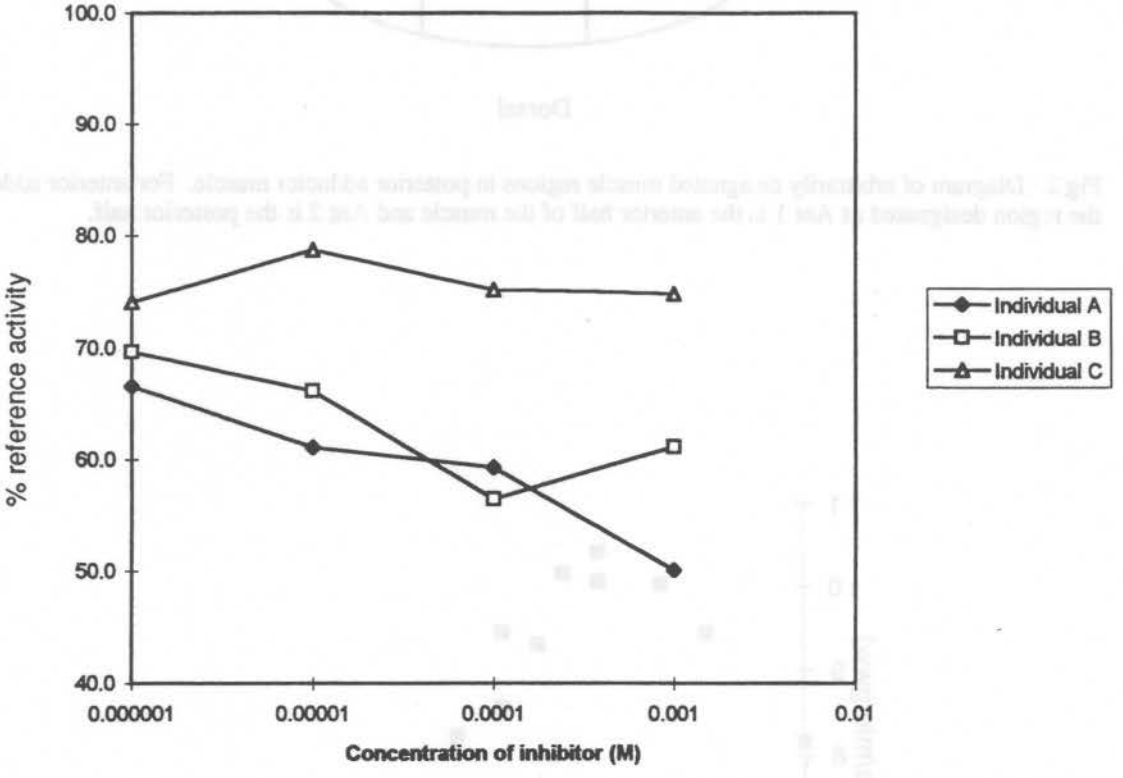

Fig. 4: BuChE inhibition assay. An aliquot of anterior adductor muscle homogenate was incubated with an equal volume of 10^{-6} to 10^{-3} M isoOMPA, a selective BuChE inhibitor, for 15 min on ice. ChE activities are expressed as a percentage of the reference activities of homogenates which had not been incubated with inhibitor.

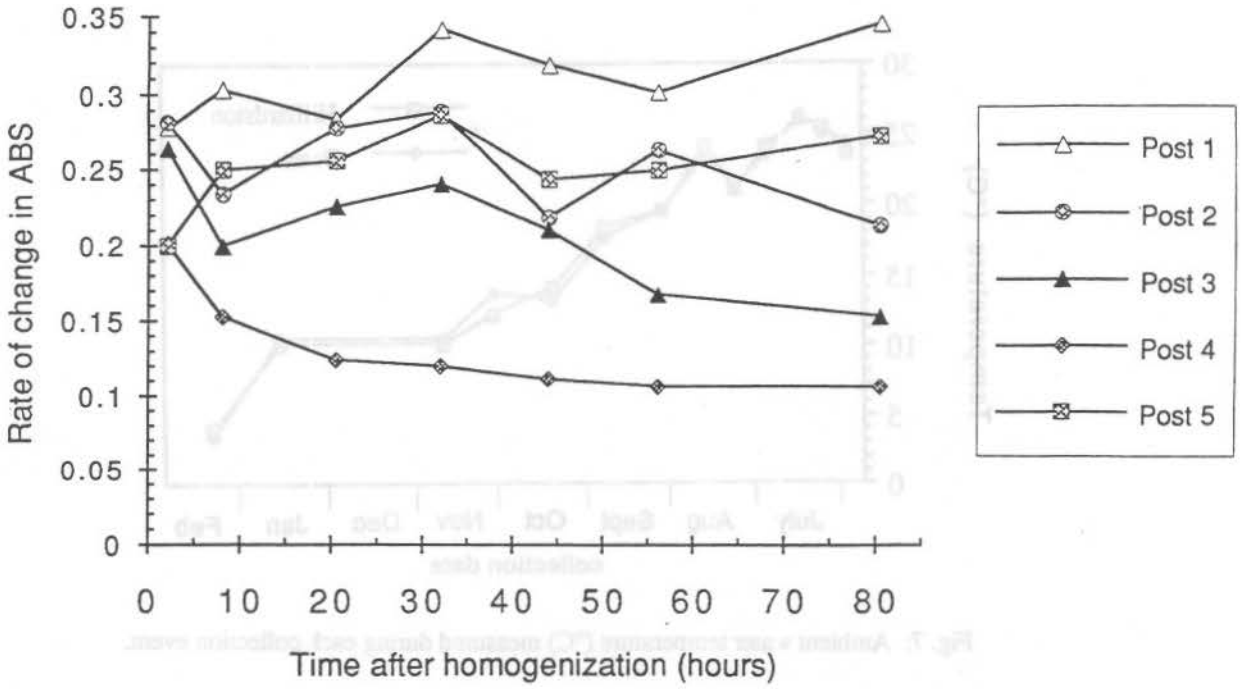

Fig. 5: Stability of ChE after homogenization and settling at various intervals at 4°C. Each data set represents a different muscle region. The rate of change in absorbance is directly proportional to ChE activity.

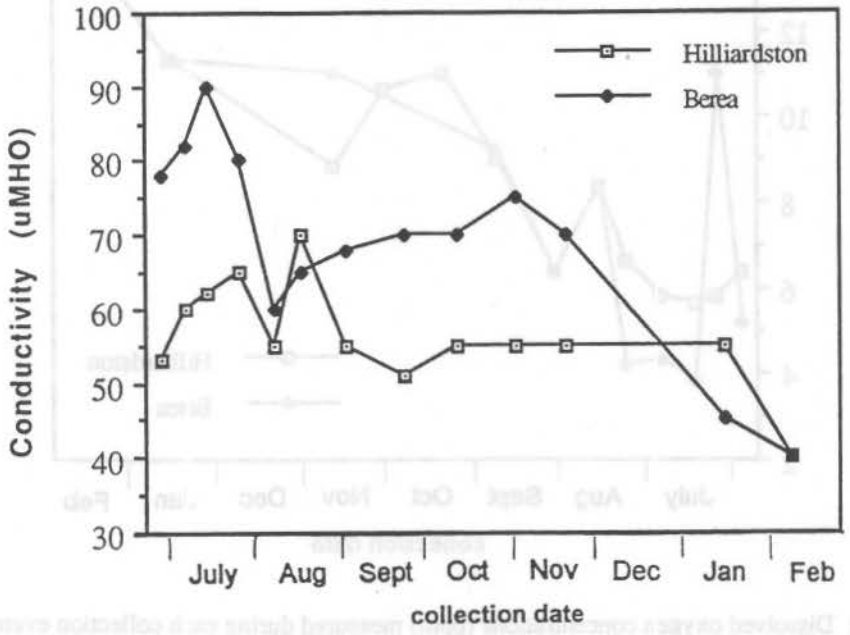

Fig. 6: Conductivity (μ MHO) of the stream water measured during each collection event.

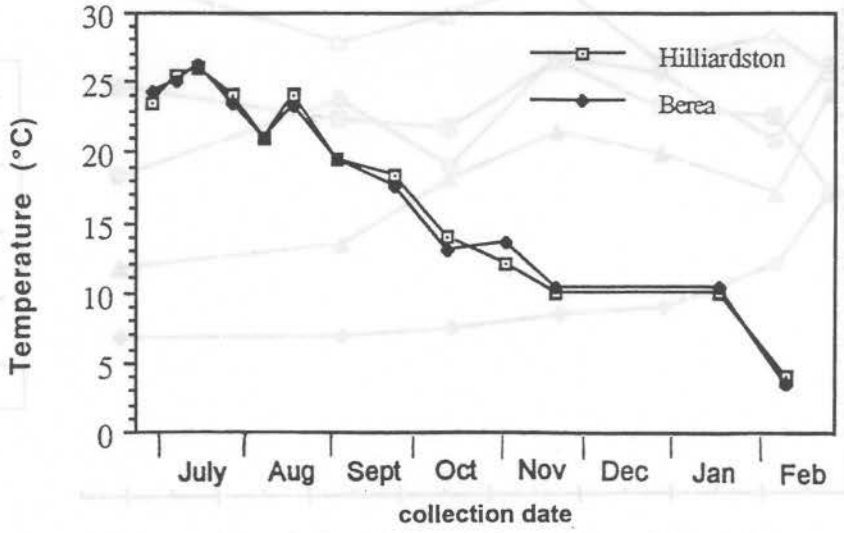

Fig. 7: Ambient water temperature (°C) measured during each collection event.

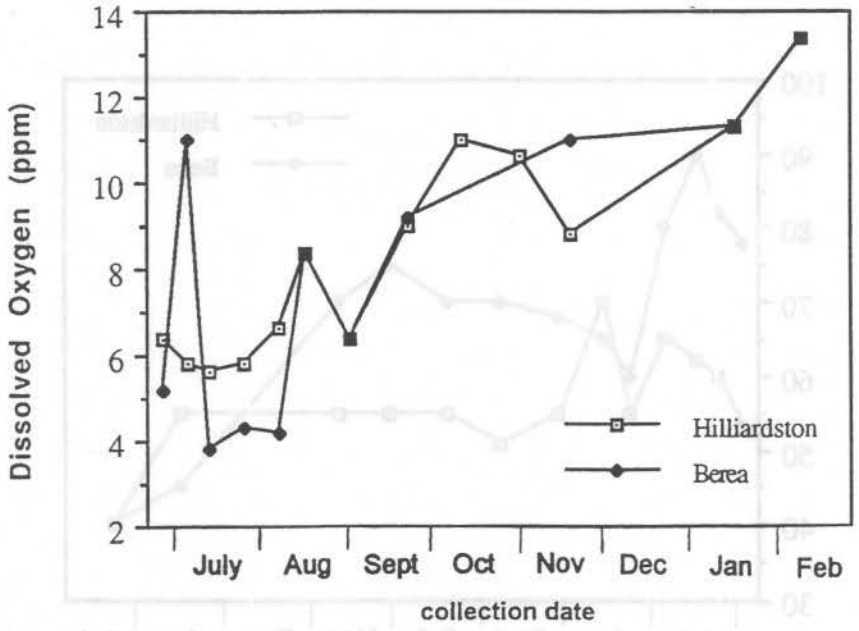

Fig. 8: Dissolved oxygen concentrations (ppm) measured during each collection event.

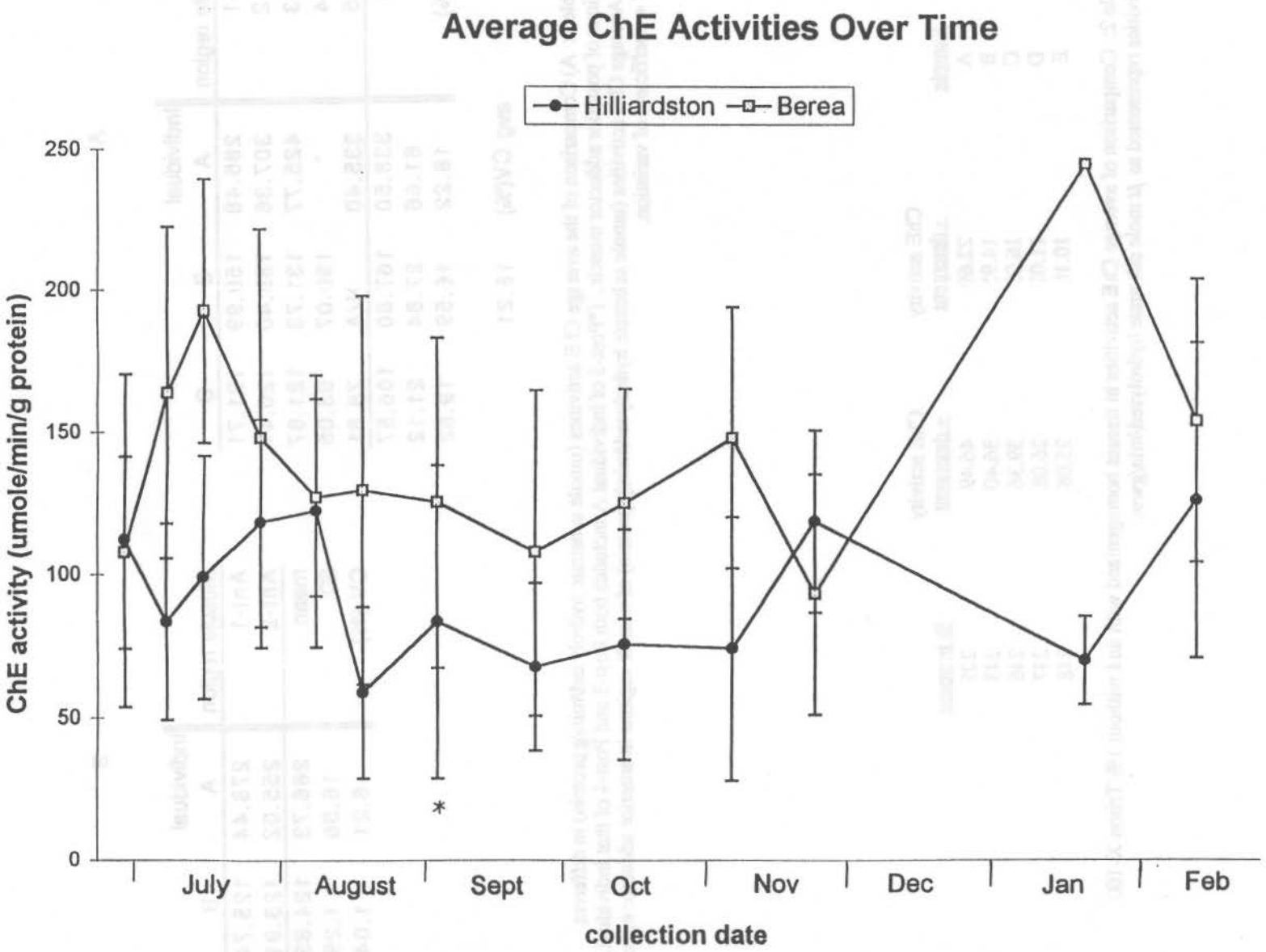

Fig. 9: Average ChE activities for the Hilliardston and Berea sites over time. For collection events through September 3, 1994, n = 10 per site per collection event. For collection events after September 3, 1994, (marked by an asterisk) n = 5. However, only one individual was collected from Berea on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

A

Muscle region	Individual A	B	C
Post-1	285.48	159.99	121.71
Post-2	307.36	189.40	120.41
Post-3	425.77	131.73	121.87
Post-4	*	190.07	93.05
Post-5	335.40	N/A	75.81
mean	338.50	167.80	106.57
SD	61.66	27.84	21.12
CV (%)	18.22	16.59	19.82
	avg CV(%)	18.21	

B

Muscle region	Individual A	B
Ant-1	278.44	125.74
Ant-2	255.02	123.91
mean	266.73	124.83
SD	16.56	1.29
CV (%)	6.21	1.04

Table 1: A) Comparison of the average ChE activities (umole substrate hydrolyzed/min/g protein) in different regions of posterior adductor muscle. (*Post-3 of individual A includes both Post-3 and Post-4 of that individual.) B) Average ChE activities (umole substrate hydrolyzed/min/g protein) of muscle regions in anterior adductor muscle. CV = coefficient of variation.

Sample	ChE activity - detergent	ChE activity + detergent	% increase
A	22.69	46.49	205
B	14.95	36.40	243
C	16.03	39.36	246
D	11.02	26.08	237
E	10.10	25.08	248

Table 2: Comparison of average ChE activities in tissues homogenized with and without 1% Triton X-100. Activities represented as μ mole substrate hydrolyzed/min/gww.

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).

	BEREA				HILLIARDSTON			
COLLECTION EVENT	mean	SD	CV (%)		mean	SD	CV (%)	
June 28, 1994	107.638	33.687	31.30	n = 4	112.056	58.372	52.09	n = 10
July 7, 1994	163.928	58.443	35.65	n = 10	83.486	34.230	41.00	n = 10
July 15, 1994	192.830	46.506	24.12	n = 10	99.153	42.645	43.01	n = 10
July 27, 1994	148.288	73.579	49.62	n = 10	118.439	36.436	30.76	n = 10
August 8,1994	127.397	34.739	27.27	n = 10	122.620	47.795	38.98	n = 10
August 18, 1994	129.982	68.289	52.54	n = 10	59.015	30.034	50.89	n = 10
September 3, 1994	125.639	57.994	46.16	n = 10	83.912	54.927	65.46	n = 10
September 24, 1994	108.035	57.180	52.93	n = 5	67.948	29.275	43.08	n = 5
October 13, 1994	125.309	40.374	32.22	n = 5	75.874	40.300	53.11	n = 5
November 5, 1994	148.433	45.890	30.92	n = 5	74.412	46.040	61.87	n = 5
November 23, 1994	93.611	42.141	45.02	n = 5	119.078	32.094	26.95	n = 5
January 20, 1995	245.454	N/A	N/A	n = 1	70.282	15.450	21.98	n = 5
February 13, 1995	154.799	49.938	32.26	n = 5	126.802	55.669	43.90	n = 5

Table 4: Mean ChE activities (μ mole substrate hydrolyzed/min/g protein), standard deviations (SD), and coefficients of variation (CV) for field samples grouped by sampling event. Sampling events which revealed a significant difference in ChE activity between collection sites are indicated in italics.