# TISSUE DISTRIBUTION AND HEMOLYMPH ACTIVITY OF SIX ENZYMES IN THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*): POTENTIAL MARKERS OF TISSUE INJURY

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# TISSUE DISTRIBUTION AND HEMOLYMPH ACTIVITY OF SIX ENZYMES IN THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*): POTENTIAL MARKERS OF TISSUE INJURY

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***ABSTRACT*** Biochemistry panels are used to help identify tissue injury (e.g., because of inflammation, trauma or hypoxia) in human and veterinary medicine in part, by detecting increased enzyme activity in serum or plasma after release from damaged tissues. To determine if a similar approach can be used in *Homarus americanus*, activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GD), sorbitol dehydrogenase (SDH), amylase (AMY) and lipase (LIP) were measured in tissue homogenates of heart, hepatopancreas, abdominal muscle, proximal intestine, antennal gland, hemocyte lysate supernatant and hemolymph plasma and serum. Activities of ALT and AST were significantly higher in serum than plasma, which was attributed to release of enzymes from hemocytes during coagulation. Reference intervals calculated for plasma enzyme activity at ambient holding temperatures of 2°C to 4°C and 15°C were quite similar. Plasma enzyme activity was not a sensitive test for detecting infection with *Aerococcus viridans* (gaffkemia) during an experimental trial.

***KEY WORDS:*** lobster, *Homarus americanus*, enzyme, hemolymph

## INTRODUCTION

The lobster fishery is a multimillion-dollar industry in Atlantic Canada and the northeastern USA. The landed value of the ~42 K mt harvest in Atlantic Canada in 2004 is estimated at ~$525,077 K (Cdn)[1]. The overall economic impact to the region of this vital industry is far greater. Although much of the harvest is sold or processed immediately, a portion is held live, for several weeks to months, in lobster pounds for sale as live product at a later date. Significant losses to disease can occur during these periods. Consequently, being able to assess the health status of these animals, as a means of selecting those best suited for storage, is very beneficial.

Current methods of health assessment in *Homarus americanus* (H. Milne Edwards 1837) and many other crustaceans include: physical examination to determine vigor; total hemocyte counts (THCs); estimating total hemolymph protein concentration using refractometry ($TP_{rf}$) and hemolymph culture to detect infective agents (bacteria, protozoa). Evaluation of the cell-free fractions of hemolymph, i.e., plasma and serum, in *H. americanus* has included electrolyte and metabolite levels (Mercaldo-Allen 1991, Mercaldo-Allen et al. 1994) and enzyme activity (Speare et al. 1996). Evaluation of hemolymph enzyme activity in mussels and oysters has been suggested as a nonlethal way of assessing overall health, and possibly immune function, as in human and veterinary medicine (Culloty et al. 2002, Gustafson et al. 2005).

Clinical enzymology can be thought of as the "application of the science of enzymes to the diagnosis of disease" (Moss & Henderson 1999). The level of enzyme activity detected in the blood is related to the rate of release or production of enzyme(s) from tissues and their clearance from the circulation (Moss & Henderson 1999). Some enzymes are relatively tissue specific, being found in only one or a few tissues, whereas others can be more widely distributed among several tissues (Duncan et al. 1994a, Kramer & Hoffmann 1997, Moss & Henderson 1999). There will usually be low levels of enzymes in the blood caused by steady-state release from tissues. These values are used to calculate reference intervals, or "normal ranges," for a defined (age, sex, race, geographic distribution, disease status, etc.) population. These reference intervals are then used as a basis for comparison with individuals or groups under study. When the tissue is injured (trauma, toxins, inflammation, hypoxia) the cellular integrity will be compromised and more enzyme will be released into the circulation, exceeding the upper limit of the previously established reference interval (Kramer & Hoffmann 1997, Moss & Henderson 1999). The degree of increase will be determined by the amount of tissue injured, the severity of the injury, and the clearance rate of the enzyme from the circulation.

The purpose of the this investigation was to determine if the principles of clinical enzymology could be applied to *H. americanus*. This required: (1) determining the tissue distribution of a selected group of enzymes; (2) determining the preferred sample type, plasma or serum; (3) establishing hemolymph reference intervals for enzyme activities in a defined population and (4) examining if hemolymph enzyme activity was affected by a systemic bacterial infection, gaffkemia.

The activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GD), amylase (AMY) and lipase (LIP) was determined in homogenates of hepatopancreas, heart, abdominal muscle, proximal intestine, antennal gland, hemocyte lysate supernatant (HLS) and hemolymph plasma and serum. Enzymes were selected for the biochemistry panel based on the availability of commercial diagnostic test kits compatible with an automated biochemistry analyzer and their known or presumed presence in hepatopancreas and muscle (Claybrook 1983, Biesiot & Capuzzo 1990)—tissues similar to those contributing significantly to serum and plasma enzyme activity in vertebrates (Kramer & Hoffmann 1997, Moss & Henderson 1999). Clotting in *H. americanus* and other crustaceans involves hemocyte lysis (Martin & Hose 1995). Consequently, HLS was evaluated for its possible contribution to serum enzyme activity. The proximal intestine, antennal gland, and heart were included for completeness. Gaffkemia, a generally fatal infectious disease caused by the Gram positive bacterium *Aerococcus viridans*, can cause significant economic losses in the industry (Håstein & Roald 1977, Stewart 1980, Menard & Myrand 1987). Early detection could provide critical informa-

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tion for management decisions and help minimize postharvest losses.

## MATERIALS AND METHODS

### *Animals and Holding*

#### Tissue Enzyme Distribution Study

Four (3 female, 1 male) hard shell, southwest Nova Scotia inshore lobsters weighing 404–499 g were obtained from a commercial pound (Clearwater Fine Foods Inc., Halifax, NS, Canada). The animals were held in a recirculating artificial sea water (ASW)(Instant Ocean, Aquarium Systems Inc., Mentor, OH, USA) system maintained between 2°C to 4°C for up to 12 d. Lighting was set at a 14 h/10 h low light (<3 lux)/dark cycle. Animals were not fed during this period.

#### Establishment of Reference Intervals and Gaffkemia Trial

Thirty male southwest Nova Scotia offshore lobsters weighing 597–791 g were obtained from a commercial source (Clearwater Fine Foods Inc., Halifax, NS, Canada). The animals were held in a recirculating ASW (Instant Ocean, Aquarium Systems Inc., Mentor, OH, USA) system maintained between 2°C to 4°C for 9 d then moved to a second recirculating ASW system at 15°C. Lighting was set at a 14 h/10 h low light (<3 lux)/dark cycle. Animals were offered a pelleted lobster diet (Castell Aquaculture Nutrition Consulting, St Andrews, NB, Canada) every other day when at 15°C.

### *Biochemical Analyses*

All samples for biochemistry panels were analyzed on the Hitachi 917 automated biochemistry analyzer (Roche Diagnostics Corporation, Indianapolis, IN, USA) within three hours of collection. Assay kits for GD, AST, ALT and total protein (TP) were obtained from Roche Diagnostics Corporation, Indianapolis, IN, USA. Assay kits for LIP and AMY were obtained from Diagnostic Chemicals Limited, Charlottetown, PE, Canada.

Assay precision was determined by measuring enzyme activity in 20 aliquots of two samples, one with high enzyme activity and one with low activity. The coefficient of variation (CV) was calculated for each sample.

### *Tissue Enzyme Distribution Study*

Hemolymph samples were collected (22 G needle) from the ventral sinus 2 d after arrival as part of a health screen. A 0.5-mL sample was collected into 4.5 mL of formalin-containing anticoagulant (28.4 g/L NaCl, 8.7 g/L $MgSO_4 \cdot 7H_2O$, 5.5 g/L $MgCl_2 \cdot 6H_2O$, 1.5 g/L $CaCl_2 \cdot 2H_2O$, 0.7 g/L KCl, 0.5 mL Tween 80, 1.25 mL/L stock [37% to 40%] formaldehyde solution, pH 7.6, Stewart et al. 1967) for determination of THCs using a Neubauer hemocytometer. Whole hemolymph (no anticoagulant) was collected for $TP_{rf}$ estimation using a temperature compensated refractometer (Shilac ATC PUR1410, Japan) and inoculation of culture media to detect *A. viridans* (0.5 mL in 4.5 mL phenyl ethyl alcohol [PEA] broth at 28°C for 48 h, in duplicate, Stewart 1972) and *Anophryoides haemophila* (0.5 mL into 4.0 mL of modified ciliate culture medium at 4°C for 7 d, Messick & Small 1996). Pleopods were collected for molt staging (Aiken 1973). Animals were processed after confirmation of negative culture results and THC > $10 \times 10^9$ cells/L and $TP_{rf}$ > 50 g/L.

Immediately prior to processing, a terminal hemolymph collection (approximately 30 mL/lobster) was performed to obtain hemocyte pellets for HLS preparation. The supernatant (plasma) was removed after centrifugation (×3300 *g*, 15 min at 4°C) and the pellet rinsed with sterile distilled water. Lobsters were euthanized by severing the ventral nerve cord in front of the walking legs. Samples of hepatopancreas, heart, antennal gland, proximal intestine and abdominal muscle were collected and immediately placed on ice. The contents of the intestinal lumen were removed by gentle rinsing with distilled water. Tissues (≤0.5 g) were then minced with a scalpel blade after addition of 5 mL of sterile distilled water, transferred to a glass piston homogenizer and ground until no large particles remained. Samples were left to lyse, refrigerated, for 1 h. Tissue homogenate supernatants were prepared by centrifugation (×15,000 *g*, 20 min, at 4°C) and assayed for enzyme activity and TP (biuret method) concentration. Enzyme activity per gram of total protein was calculated to standardize the results among tissues.

### *Comparison of Plasma and Serum Samples*

Hemolymph was collected (22 G needle, no anticoagulant) from the ventral sinus of the four lobsters used for the tissue enzyme distribution study. Plasma was prepared from one 1.8 mL aliquot. Serum was prepared by allowing refrigerated 1.8 mL aliquots to clot. After 5 h and 22 h, the samples were broken up and centrifuged (×5000 *g*, 5 min, at 4°C) and the supernatants collected and submitted for biochemistry panels.

### *Establishment of Reference Intervals*

Hemolymph samples for THCs, $TP_{rf}$, biochemistry panels and inoculation of PEA were collected from the ventral sinus of 30 male, hardshell lobsters 2 d and 6 d after their arrival (2°C to 4°C) and again after being held at 15°C for 4 d. Pleopods for molt staging (Aiken 1973) and hemolymph for ciliate medium (Messick & Small 1996) inoculation were only collected at the first sampling. Reference intervals were calculated for the latter two sample collections using results from intermoult animals with THC >5 × $10^9$ cells/L and negative hemolymph cultures.

### *Gaffkemia Infection Trial*

#### *Aerococcus viridans* Inoculate Preparation

A thawed aliquot of a first passage subculture of a field isolate of *A. viridans* Type 3 (api 20 Strep, bioMérieux Canada Inc., St. Laurent, PQ, Canada), stored at −80°C in 2% skim milk culture broth (Bacto skim milk, DIFCO Laboratories, Detroit, MI), was grown on sheep blood (5%) agar (BA) (Oxoid, Inc. (Canada), Nepean, ON) at 28°C for 24 h and used to inoculate 25 mL of sterile trypticase soy broth (TSB). Broth cultures were incubated overnight at 28°C and the optical density was read at 420 nm. The suspension was washed (×3600 *g*, 10 min, at 4°C) twice with sterile 3% NaCl and an aliquot diluted to an expected concentration of $2 \times 10^6$ colony forming units (CFU)/mL. Colony counts were performed on ×25 μm aliquots of serial dilutions cultured on BA at 28°C after 48 h of incubation to confirm inoculate dose.

The supernatant from a 24 h TSB culture of *A. viridans* was submitted for a biochemistry panel.

#### Experimental Protocol

Thirty male lobsters were acclimatized at 2°C for 10 d and then transferred into a new system at 15°C for 7 d. Hemolymph samples

were collected from the ventral sinus for determination of THCs, $TP_{rf}$ and biochemistry panels and a few drops of whole hemolymph were used to inoculate BA, incubated at 28°C, for detection of *A. viridans* bacteremia. Twenty lobsters with THC > $10 \times 10^9$ cells/L, and negative culture results were selected and randomly assigned into treatment and control groups. Control group lobsters were moved into a replicate system in the same room. Lobsters in the treatment group were inoculated (intra-abdominal sinus injection, 25 G needle) with *A. viridans* at ~$1 \times 10^6$ CFU/kg. Control group lobsters received a similar volume of sterile 3% NaCl. One sentinel lobster (no injection) was kept in each tank.

Hemolymph was collected daily for determination of THCs, $TP_{rf}$, biochemistry panels and BA inoculation. A direct smear (DS) of hemolymph was also made and examined by direct light microscopy to detect bacteremia. The study was terminated after 5 d because all treatment group lobsters were bacteremic on DS exam.

Lobsters were euthanized by KCl injection (Battison et al. 2000). Post mortem examinations were performed. The hepatopancreas and antennal gland were sampled with sterile swabs (BBM CultureSwab Plus, Becton Dickinson, Basel, Switzerland) for culture on BA at 28°C for 48 h. Representative samples of hepatopancreas, proximal intestine, antennal gland, muscle, gonad and ventral nerve cord were collected and fixed in 1G:4F fixative (Howard & Smith 1983) and processed routinely for hematoxylin and eosin staining of paraffin sections.

### *Statistical Analyses*

Statistical analyses were performed using the Minitab release 13 software package (Minitab Inc., State College, PA, USA) and Microsoft Excel 2002 (Microsoft Corporation, USA).

## RESULTS

### *Precision Study*

Precision results at the lower activity levels found in plasma were generally moderate to poor, ranging from 5% to 24% (Table 1). The value for SDH was very high (245%) as all but one of the 20 replicates returned a value of 0 U/L. Precision at the higher activity levels found in serum was acceptable, being below or near 5% in all cases.

**TABLE 1.**

**Precision results for six enzymes* in plasma or serum, representing lower and higher activity levels respectively, in *Homarus americanus*. An aliquot of each sample was assayed 20 times.**

| | AMY | LIP | SDH | ALT | AST | GD |
|---|---|---|---|---|---|---|
| Plasma | | | | | | |
| Range (U/L) | 0–0 | 9–15 | 0–8 | 14–33 | 11–14 | 11–13 |
| CV** | – | 16% | 245% | 24% | 9% | 5% |
| Serum | | | | | | |
| Range (U/L) | 0–0 | 6–8 | 0–0 | 220–224 | 111–129 | 13–20 |
| CV | – | 7% | – | 2% | 4% | 7% |

* Abbreviations: AMY, amylase; LIP, lipase; SDH, sorbitol dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.

** CV, coefficient of variation = standard deviation/mean * 100.

### *Tissue Enzyme Distribution*

The hepatopancreas was the exclusive source of AMY in all but one lobster where it was also detected in the proximal intestine (Table 2). Moderate to high levels of AST and ALT were found in all tissues with the heart containing the most activity (Table 2). The highest GD activity was found in the heart with only low to moderate levels recovered from other tissues (Table 2). LIP activity was low, but primarily isolated from proximal intestine preparations (Table 2). SDH activity was low and widely distributed.

### *Comparison of Plasma and Serum*

There was minimal or no AMY, LIP, or SDH in the plasma or serum samples. There were notable differences between the plasma and serum samples for AST, ALT and GD in all lobsters. In all instances, serum values were higher than plasma at both time periods (Table 3). The amount of increase varied with the individual lobster and the enzyme. Changes in ALT were usually the most marked, whereas GD was relatively stable. Serum enzyme activity at 22 h tended to be less than at 5 h.

### *Screening Samples and Establishment of Reference Intervals*

Enzyme activity collected 2 d after arrival was quite variable with some lobsters having AST and/or ALT activities up to 10 fold greater than the values obtained from the same animals only 4 d later (Table 4). A similar, but much less dramatic, finding occurred with LIP (Table 4).

Enzyme activity in the two data sets used for reference interval calculation had a nonGaussian distribution except for GD at 15°C. After removal of outliers, results from 25 of the 30 lobsters sampled after 6 d at 2°C, and 26 of the lobsters sampled after 4 d at 15°C were used to calculate the plasma reference intervals using percentiles (Table 4).

### *Gaffkemia Infection Trial*

Animals received inocula of ~$1.5 \times 10^6$ CFU/kg based on 48 h colony counts. A contaminant (*Acinetobacter* sp.) was detected in the inoculate suspension but not recovered from any lobsters. Bacteremia was detected on hemolymph culture in nine treatment group lobsters on postinoculation Day 2 and in all lobsters by Day 3. Four *A. viridans* colonies were detected in one control group lobster on Day 5 only. Bacteria were visible on DS in eight treatment group lobsters by Day 4 and in all by Day 5. A heavy growth of *A. viridans* was recovered from the hepatopancreas and antennal gland of all treatment group animals but no control group lobsters. Very low numbers of mixed bacteria were recovered from the antennal gland of nine, and from the hepatopancreas of three, control group lobsters.

THCs showed an initial increase Days1–3 in treatment group lobsters followed by a decrease to ~$2 \times 10^9$ cells/L by Day 5 in most (8/10) lobsters as seen in previous studies (Stewart et al. 1969, Stewart et al. 1983, Battison et al. 2004). A progressive decrease in THC (<$5 \times 10^9$ cells/L) was also noted in 5 of 10 control group lobsters. $TP_{rf}$ decreased by 10% to 15% of initial values by the end of the study in all, but one control (49%), lobsters.

For most lobsters, enzyme activities stayed within the previously established reference intervals (Fig. 1). There was a tendency for LIP to decrease over the course of the trial for both groups. A few animals (treatment and control groups) had minimal increases in ALT. Mild to moderate increases in GD were ob-

**TABLE 2.**

**Activity of six enzymes* in six tissues expressed as U/gram of total protein (biuret method) in tissue homogenate supernatants from four lobsters (*Homarus americanus*). Results presented as mean and (range) of values obtained.**

| | AMY | LIP | SDH | ALT | AST | GD |
|---|---|---|---|---|---|---|
| Heart | 0 (0–0) | 1 (0–1) | 2 (2–2) | 1425 (1311–1956) | 2243 (1593–2970) | 573 (358–602) |
| Antennal gland | 0 (0–0) | 0 (0–1) | 3 (2–3) | 246 (192–341) | 562 (501–624) | 16 (7–30) |
| Proximal intestine | 0 (0–4) | 3 (2–5) | 1 (1–1) | 518 (405–678) | 314 (293–334) | 67 (62–70) |
| Hepatopancreas | 13 (7–19) | 0 (0–1) | 4 (2–4) | 134 (5–342) | 829 (706–1438) | 6 (3–10) |
| Abdominal Muscle | 0 (0–0) | 0 (0–0) | 1 (0–1) | 157 (116–180) | 449 (373–507) | 32 (14–45) |
| Hemocyte lysate | 0 (0–0) | 1 (1–1) | 0 (0–0) | 323 (220–397) | 101 (46–138) | 52 (45–60) |

* Abbreviations: AMY, amylase; LIP, lipase; SDH, sorbitol dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.

served in 3 lobsters (2 treatment and 1 control). Elevations in SDH were limited to one treatment group lobster, which also had moderate increases in AST, ALT and GD and two control group lobsters—one of which had moderately elevated GD. Essentially no enzyme activity was detected in the TSB culture supernatant.

The main gross finding in the treatment group lobsters was the presence of multifocal to diffuse, white, miliary, spots on the antennal gland. All of the control group lobsters showed variable degrees of pigmentation at the base of the gill filaments. Two treatment group lobsters had mild, focal, white discoloration of abdominal muscle segments (necrosis) near hemolymph collection sites.

Histological findings in the treatment group were consistent with gaffkemia (Johnson et al. 1981, Battison et al. 2004). Coccoid bacteria in tetrads were present in the hemolymph spaces of all tissues. Small hemocyte nodules, some with phagocytosed cocci, were noted in low to moderate numbers in most tissues examined.

There was moderate to marked, multifocal to diffuse, involvement of the fixed phagocytes in the connective tissue of the hepatopancreas. The cells were enlarged and vacuolated, containing large numbers of bacteria and often surrounded by hemocytic nodules (Fig. 2). Deposition of a red-orange, hyaline material was noted in some nodules (Fig. 2). The antennal glands were similarly affected with multifocal to coalescing septic, variably sized, hemocytic nodules. Moderate, focal, myonecrosis was confirmed in one lobster.

All control group lobsters had a moderate to severe necrotising, nonseptic, melanizing, branchitis. Large hemocyte aggregates were present in many gill filaments sometimes filling the vascular channels, similar to a thrombus. Adjacent tissue was uniformly lightly eosinophilic (necrotic) in some sections. Infectious agents were not identified. Neither *A. viridans* nor lesions typical of gaffkemia were found in the one control group lobster which had a single positive hemolymph culture.

## DISCUSSION

Clinical chemistry is an important diagnostic tool in human and veterinary medicine. The purpose of this investigation was to determine if this tool could also be applied to lobsters, and potentially other crustaceans. To interpret hemolymph enzyme activity, it was necessary to determine the tissue distribution of the enzymes in lobsters. This was important because enzymes that are tissue spe-

**TABLE 3.**

**Activity of three enzymes* (ALT, AST, GD) measured in plasma (processed immediately) and serum (allowed to clot for 5 and 22 h at 4°C) samples from four lobsters (*Homarus americanus*). Fold increase from plasma is indicated in parentheses.**

| | Plasma | Serum @ 5 h | Serum @ 22 h |
|---|---|---|---|
| ALT (U/L) | 31 | 237 (7.6×) | 106 (3.4×) |
| | 18 | 286 (15.8×) | 102 (5.6×) |
| | 19 | 171 (9.0×) | 167 (8.8×) |
| | 15 | 399 (26.6×) | 351 (23.4×) |
| AST (U/L) | 38 | 185 (4.8×) | 90 (2.3×) |
| | 24 | 206 (8.5×) | 85 (3.5×) |
| | 16 | 111 (6.9×) | 124 (7.7×) |
| | 15 | n/a** | 164 (10.9×) |
| GD (U/L) | 11 | 18 (1.6×) | 15 (1.3×) |
| | 10 | 18 (1.8×) | 15 (1.5×) |
| | 9 | 13 (1.4×) | 16 (1.7×) |
| | 19 | 24 (1.2×) | 26 (1.3×) |

* Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.
** Not available.

**TABLE 4.**

**Plasma enzyme* activity in 30 lobsters (*Homarus americanus*) two days after arrival expressed as mean (and range) and reference intervals calculated after 6 d at 2–4°C and 4 d at 15°C.**

| | 2 d @ 2–4°C (*n* = 27)*** | Reference Interval** (2–4°C) (*n* = 25)**** | Reference Interval** (15°C) (*n* = 26)***** |
|---|---|---|---|
| AMY (U/L) | 0 (0–0) | 0–0 | 0–0 |
| LIP (U/L) | 8 (5–20) | 0–4 | 4–9 |
| SDH (U/L) | 0 (10–14) | 0–19 | 0–0 |
| ALT (U/L) | 28 (12–300) | 6–24 | 6–21 |
| AST (U/L) | 25 (8–319) | 5–16 | 4–12 |
| GD (U/L) | 17 (7–31) | 6–26 | 10–34 |

* Abbreviations: AMY, amylase; LIP, lipase; SDH, sorbitol dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GD, glutamate dehydrogenase.
** $5^{th}$–$95^{th}$ percentiles.
*** Three samples clotted, data not available.
**** Five outliers removed.
***** Four outliers removed.

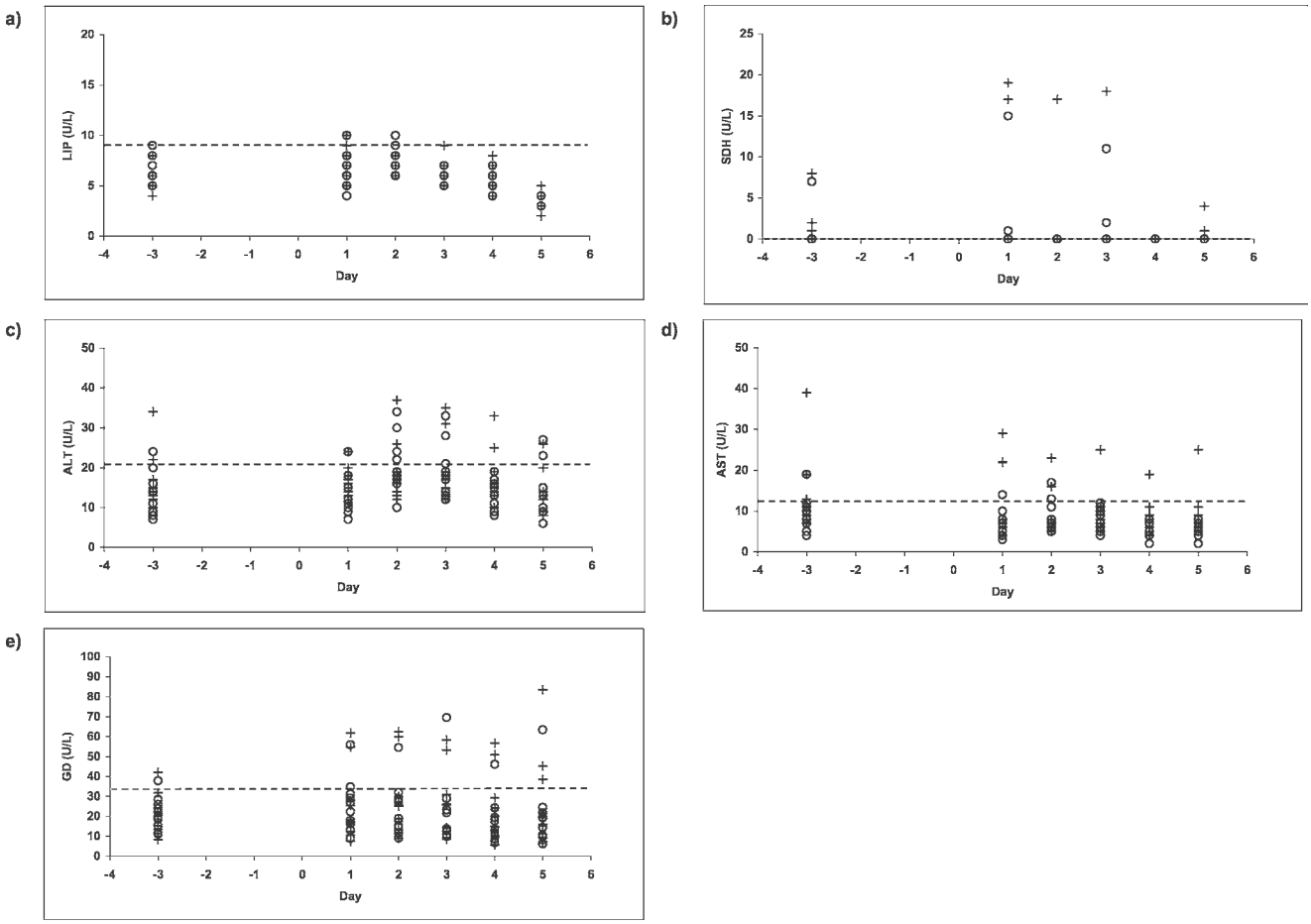


**Figure 1. Plasma activity of: (a) lipase (LIP), (b) sorbitol dehydrogenase (SDH), (c) alanine aminotransferase (ALT), (d) aspartate aminotransferase (AST) and (e) glutamate dehydrogenase (GD) for lobsters (*Homarus americanus*) inoculated with *Aerococcus viridans* bacteria (+; $n = 10$) or 3% sterile NaCl (○; $n = 10$). Samples were collected prior (Day 3) to inoculation on Day 0 and daily during the trial (Days 1–5). The dashed line represents the upper limit of the reference interval for plasma activity for each enzyme.**

cific in one species will not always be so in others (Duncan et al. 1994a).

Biochemistry panels in veterinary medicine may include enzymes of skeletal muscle (creatinine kinase (CK), AST, ALT), hepatocellular (AST, ALT, SDH and GD) and pancreatic (AMY, LIP) origin as a means to assess damage to, or dysfunction of, these tissues in addition to data on electrolyte and metabolite concentrations (Kramer & Hoffmann 1997, Moss & Henderson 1999). Enzymes present in the kidneys and intestine are not generally found in the blood in vertebrates (Kramer & Hoffmann 1997, Moss & Henderson 1999). Instead, when these tissues are damaged, enzymes are believed to be released into the urinary space or intestinal lumen rather than into the circulation (Kramer & Hoffmann 1997). A similar situation is expected for the antennal gland and intestinal tissues in the lobster. When injury to the heart is suspected in vertebrates, the results of more specialized testing (e.g., isoenzyme determination) are combined with other clinical information to differentiate cardiac from general muscle injury because the enzyme composition of the tissues is very similar (Moss & Henderson 1999).

Overall, tissue enzyme activity in lobsters in this study resembled the distribution seen in other animals (Moss & Henderson 1999), with some exceptions. The low activity of GD recovered from the hepatopancreas was surprising because this mitochondrial enzyme is central to ammonia metabolism and is known to be present in crustaceans (Claybrook 1983). An inhibitor may be responsible. GD activity could be detected in the isolated mitochondrial fraction, but not in homogenates, of the hepatopancreas in the crayfish (*Orconectes limosus*) (Claybrook 1983). A similar effect could account for the results obtained in this study. Plasma from apparently healthy lobsters often contains moderate levels of GD—presumably of muscle and/or hepatopancreatic origin.

AST and ALT are commonly used as indicators of hepatocellular injury in vertebrate species (Duncan et al. 1994a, Moss & Henderson 1999). Skeletal muscle injury is usually indicated by increases in CK and AST, but may also be accompanied by increased ALT in severe disease (Valentine et al. 1990, Kramer & Hoffmann 1997, Moss & Henderson 1999). For this reason, it is useful to have at least one enzyme that is considered tissue specific. CK, as a muscle-specific enzyme, serves this purpose in vertebrates (Kramer & Hoffman 1997, Moss & Henderson 1999). The equivalent enzyme in lobsters is arginine kinase (AK) (Horney et al. 2001). Unfortunately, neither an AK assay kit nor the reagents necessary for modifying the CK assay were available at the time of this study. Consequently, plasma AST and ALT activity in lobsters should be considered to be of either hepatopancreas or muscle origin.

SDH can be used as a specific indicator of hepatocellular injury

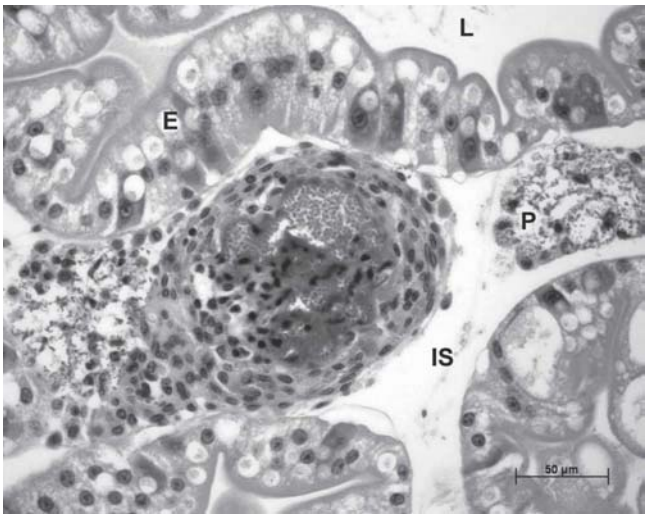


**Figure 2.** ***Homarus americanus*****, hepatopancreas. Hemocyte nodule in the intertubular space (IS). The nodule contains numerous cocci (*Aeroccus viridans*) in the center. The smaller group of cells at the upper right is a group of fixed phagocytes (P) with intracellular cocci. The hepatopancreatic epithelial cells (E) appear unaffected. The tubule lumen (L) is indicated.**

(Duncan et al. 1994a, Moss & Henderson 1999). The relatively low SDH activities detected in multiple tissues in this study could indicate that either there is very little of this enzyme in these tissues, an inhibitor is present, or that the assay conditions (reaction temperature, substrate, etc.) were not optimal. Plasma SDH activity was often low or had poor repeatability. These findings suggest that SDH, under current assay conditions, may not be a useful enzyme for inclusion in lobster biochemistry panels. Further testing under different disease conditions will be required to confirm this.

Lipase is generally used as an indicator of pancreatic injury in vertebrates (Duncan et al. 1994b, Moss & Henderson 1999). Given the digestive function of the hepatopancreas, significant levels were expected in this organ as reported previously (Brockerhoff et al. 1967, Brockerhoff et al. 1970, Hoyle 1973, Biesiot & Capuzzo 1990). However, only relatively low activity was detected in the hepatopancreas with slightly higher activity in the proximal intestine in most lobsters in the current study. This discrepancy could be because of the different assay methodologies, or possibly an inhibitory substance. Differences in reporting enzyme activity among the studies make it impossible to compare the results. The presence of LIP activity in the intestinal preparations could indicate an additional location (i.e., enterocytes) for this enzyme or, incomplete removal of the lumen contents during sample preparation

Detection of moderate levels of plasma LIP activity may be an indicator of recent feeding. Plasma LIP activities decreased while lobsters were held, fasted, at 2°C and increased when the animals were transferred to 15°C and fed. Refeeding after a period of starvation has been suggested as the cause of increased LIP activity in gastric fluid in *H. americanus* (Hoyle 1973). Plasma LIP activity decreased again in both groups as the trial progressed. Lobsters are reported to become inappetent shortly after infection with *A. viridans* (Stewart et al. 1972)—possibly a response to the inflammation associated with this disease. The branchitis may have caused a similar effect in the lobsters in the control group. Unfortunately, food intake was not monitored closely enough in either group to confirm this hypothesis.

Amylase activity has been detected in hepatopancreas homogenates and gastric fluid in *H. americanus* (Wojtowicz & Brockerhoff 1972, Hoyle 1973, Biesiot & Capuzzo 1990). The hepatopancreas was the only tissue to consistently contain any significant AMY activity in this study. Because AMY activity was not detected in plasma samples from apparently healthy lobsters, this may indicate that plasma AMY will prove to be a sensitive and specific indicator of hepatopancreatic epithelial cell injury.

The reference intervals calculated for the two temperatures were quite similar, suggesting little effect of temperature. Whereas the intervals for male lobsters at 2°C to 4°C are likely an accurate reflection of lobsters in their natural surroundings, the intervals for 15°C may not be, because these lobsters were rapidly brought to this temperature under artificial conditions. Sampling lobsters that have gone through natural acclimation, with its attendant physiological adaptations, including possible induction of enzymes more suited to these higher temperatures, will be required to determine the validity of the current findings. Obtaining samples from a larger population of animals of both sexes at different molt stages from different fishing areas and during different seasons, is required to assess the possible effects of these variables.

Plasma is the preferred sample for measuring hemolymph enzyme activity. In the four animals tested, activities of ALT, AST and to a lesser degree, GD, were higher in serum than plasma. Activity of all three of these enzymes was found in the HLS. Thus, the serum activity is presumed to come from the hemocytes that lyse as part of the coagulation mechanism (Martin & Hose 1995). It is likely that the amount of enzyme contributed by the hemocytes will depend on both the total and differential hemocyte counts. A recent publication examined enzyme activity in hemolymph serum (Dove et al. 2005). It was noted that ALT and AST decreased in "warmer months," coincidental with absent hemolymph clotting. Poor clotting is frequently caused by hemocytopenia (Martin & Hose 1995)—the ALT and AST values obtained may have been an indirect indicator of the total and differential hemocyte counts rather than, or in addition to, release of these enzymes from tissues.

The benefits of using automated analyzers, with their internal standards and controls, include the relatively rapid determination of the activity of multiple enzymes in a small sample volume (~300 µL). Enzyme activity will be affected by reaction conditions such as substrate, temperature, and pH. For these reasons, reference intervals for enzyme activity are considered laboratory specific. In this study, all assays were performed at 37°C, the standard operating temperature of the automated chemistry analyzers and assay kits, although above the reported thermal tolerance (−1°C–30°C) of *H. americanus* (Lawton & Lavalli 1995). Previous work in homarid and panulirid lobsters and bivalves has detected enzyme activity when measured at 25°C and 37°C, with proteases of the spiny lobster demonstrating maximal activity at 50°C (Brockerhoff et al. 1970, Wojtowicz & Brockerhoff 1972, Hoyle 1973, Biesiot & Capuzzo 1990, Horney et al. 2001, Culloty et al. 2002, Celis-Guerrero et al. 2004, Gustafson et al. 2005).

Plasma enzyme activity appeared relatively stable when samples were refrigerated (2°C to 4°C) for up to 4 d or frozen at −20°C for 6 wk (results not presented). Stability studies were planned; unfortunately, only two samples with moderate enzyme activities were available for evaluation. The slight variations noted in these two samples may have been caused by the suboptimal

precision at these activity levels. Plasma AK had limited stability under different storage conditions and same day analysis was recommended (Horney et al. 2001).

Infection with *A. viridans* did not cause any consistent, specific, or clinically significant (>2–3× increase above upper limit of the reference interval) changes in plasma enzyme activity in this study to allow early detection of the *A. viridans* infection. The hepatopancreas is a major site of inflammation in gaffkemia, yet there was little evidence of damage to this tissue on the biochemistry panels (i.e., clinically significant increases in AMY, LIP, ALT, or AST activity). This may have occurred because the inflammation was primarily located in the connective tissues with relatively little involvement of the hepatopancreatic tubule epithelium, the presumed location of these enzymes. Increases in plasma enzyme activity may have been observed if the trial had progressed until the lobsters died of the infection when the inflammatory lesions would have been more severe.

Clinical enzymology is dynamic with hemolymph enzyme activity reflecting current conditions. Five lobsters had very high AST and ALT activity 2 d after arrival—the highest enzyme activities observed in this study. The most likely origin of these enzymes would be muscle and/or the hepatopancreas. The values in all five lobsters had decreased significantly by the next sample collection only 4 d later, suggesting a transient insult, possibly related to trauma and/or hypoxia experienced during shipping. Three of these five lobsters (1 control and 2 treatment) showed progressive increases in GD accompanied by mild increases in AST and/or ALT during the gaffkemia trial. Whether the increase in GD was related to the previously elevated AST and ALT, or to some underlying process exacerbated by the *A. viridans* infection, remains speculative. There were no gross or histological lesions unique to any of these five lobsters at necropsy to account for the enzyme changes.

## CONCLUSIONS

This study has identified the tissue distribution and established plasma reference intervals in *H. americanus* for ALT, AST, GD, AMY, LIP and SDH. This information can be used as a starting point to assess the usefulness of clinical enzymology for the diagnosis of disease in *H. americanus*. More information on the stability of these enzymes under different storage conditions, factors that can affect their circulating half life, possible induction and effects of the molt cycle on plasma activity, is needed. Plasma enzyme activities are most likely a tool to be used in research settings at present. Although plasma enzyme activity did not prove to be a sensitive test for detecting gaffkemia, with further investigation one or more enzymes may be identified that can be used to assess the general health of lobsters upon arrival at pounds (e.g., screening for the presence of underlying diseases) or detecting the effects of rough handling during transport, which might affect survivability under long-term holding conditions. This information could be useful when making marketing and management decisions.

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