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Tolerance, Bioenergetics and Biochemical Composition of the Blue Crab, Callinectes Sapidus Rathbun, Exposed to the Water-Soluble Fraction of South Louisiana Crude Oil.

Shiao Yu Wang

Louisiana State University and Agricultural & Mechanical College

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TOLERANCE, BIOENERGETICS AND BIOCHEMICAL COMPOSITION OF THE BLUE CRAB, CALLINECTES SAPIIDUS RATHBUN, EXPOSED TO THE WATER-SOLUBLE FRACTION OF SOUTH LOUISIANA CRUDE OIL

The Louisiana State University and Agricultural and Mechanical Col. PH.D. 1986

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TOLERANCE, BIOENERGETICS AND BIOCHEMICAL COMPOSITION OF
THE BLUE CRAB, CALLINECTES SAPIDUS RATHBUN,
EXPOSED TO THE WATER-SOLUBLE FRACTION OF SOUTH LOUISIANA CRUDE OIL

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in
The Department of Zoology and Physiology

by
Shiao Yu Wang
B.S., William Carey College, 1977
M.S., University of Southern Mississippi, 1981
December 1986
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I am greatly indebted to my dissertation committee which is composed of Drs. Stickle, Dietz, Fleeger, Weidner and Bartlett. I am grateful for their criticisms, suggestions, counseling and encouragements. Dr. Stickle, my committee chairman, was a fine mentor and a good friend.

Many people shared their knowledge and permitted me to use equipment and/or supplies belonging to them. Ken Stuck, Gulf Coast Research Laboratory, Mississippi, taught me much about crab biology and helped me with the field work. Dr. Stanley Rice, National Marine Fisheries Service, NOAA, Auke Bay, Alaska gave me the opportunity to visit his laboratory and learn how crude oil toxicity studies were conducted in his laboratory. Dr. Judith McDowell Capuzzo, Woods Hole Oceanographic Institution, Massachusetts, allowed me to use the Iatroscan in her laboratory and provided me with research supplies. My research would have been difficult without their help and advice; their kindness and generosity will always be remembered.

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I thank the Petroleum Refiners Environmental Council of Louisiana which provided financial support for research supplies. South Louisiana crude oil was donated by the Baton Rouge Refinery of Exxon Company, USA.
FORWARD

The subject of my dissertation research is the physiological and biochemical responses of juvenile blue crabs, *Callinectes sapidus*, to the water-soluble fraction of South Louisiana crude oil. During the first phase of the project, I designed and constructed a flow-through system in the laboratory with which I could expose crabs to consistent exposure concentrations for long durations (at least 28 days). Subsequently, I determined the long-term tolerance of blue crabs to petroleum hydrocarbons. The sublethal concentrations used later were determined using the 21-day LC$_{50}$ (the predicted hydrocarbon concentration which would kill 50% of the test organisms in 21 days) as a reference.

Bioenergetic studies were conducted to study the physiological responses of blue crabs to petroleum hydrocarbons. Bioenergetic studies integrate basic physiological rate functions and provide insight on the mode of action of pollutants on the whole organism. This was the subject of the first publication: Bioenergetics, growth and molting of the blue crab, *Callinectes sapidus*, exposed to the water-soluble fraction of South Louisiana crude oil. This paper is in press in a book titled: Pollution Physiology of Estuarine Organisms; F.J. Vernberg, W.B. Vernberg, A. Calabrese and F.P. Thurberg (eds.). The book will be published by the University of South Carolina Press, Columbia, South Carolina.

In the second paper, the possible use of the RNA:DNA and protein:DNA ratios as indicators of stress was examined. In an earlier study, I had found the RNA:DNA ratio to be a reliable and sensitive indicator of nutritional stress in juvenile blue crabs (Wang, S.Y. and
W.B. Stickle, 1986. J. Crust. Biol. 6:49-56). In the second paper, I also summarized the bioenergetic data in terms of ash free dry weight and provided correlative evidence showing the relationship between scope for growth (energy accumulated for growth and reproduction) and actual growth. The literature has been sparse in this area and it has often been assumed that changes in scope for growth are accompanied by changes in growth and/or reproduction. This paper has been submitted for publication in the journal Marine Biology.

After determining changes in energetics and growth in blue crabs as a result of exposure to crude oil, the question was how are the tissue content and constituents altered. Biochemical changes in crabs exposed to petroleum hydrocarbons were determined and is the subject of the third paper which has also been submitted for publication in Marine Biology.
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ABSTRACT

The tolerance, bioenergetics, growth, molting frequency and biochemical composition of juvenile blue crabs, *Callinectes sapidus*, exposed to the water-soluble fraction of South Louisiana crude oil were determined during a 21-d exposure period. Blue crabs were highly tolerant to petroleum hydrocarbons; the LC50 was 3927 ppb on day 21. Rates of energy intake decreased with increasing hydrocarbon concentration. Although energy expenditure rates of crabs exposed to 2504 ppb were not significantly higher than that of control crabs, they were significantly higher than those of crabs exposed to 820 and 1476 ppb. The resulting decrease in scope for growth was thus due to both a decrease in energy intake at higher exposure concentrations and a dose-dependent increase in energy expenditure. Molting was delayed in crabs exposed to 1476 and 2504 ppb. Crab growth was significantly reduced in a dose-dependent manner. The correlation between scope for growth and tissue growth was highly significant, indicating a bioenergetic basis for the decreased growth in crabs exposed to petroleum hydrocarbons. RNA content of crabs exposed to crude oil was significantly reduced on all sampling dates. Protein content of crabs exposed to crude oil was lower than that of control crabs on days 14 and 21. DNA content was not significantly different from that of control crabs, suggesting that the differences in tissue content was due to differences in cell content and not cell number. RNA:DNA and protein:DNA ratios were significantly reduced in crabs exposed to crude oil. The ratios were positively correlated with scope for growth and observed growth. The significant correlations suggest the possibility of their use as indicators of physiological condition. Significant differences in
lipid content and concentration were observed. Analysis of five lipid classes indicate that structural lipids were less affected than lipids used for energy storage among crabs exposed to crude oil. The observed changes in growth pattern and biochemical composition suggest that the pattern of energy utilization was altered. Among crabs exposed to petroleum hydrocarbons, growth in size took place despite little increase in tissue content. Available energy was used for growth with little being stored in lipid reserves.
CHAPTER ONE:
BIOENERGETICS, GROWTH AND MOLTING OF THE BLUE CRAB, 
CALLINECTES SAPIIDUS, EXPOSED TO THE WATER-SOLUBLE FRACTION 
OF SOUTH LOUISIANA CRUDE OIL

INTRODUCTION

Bioenergetic studies integrate basic physiological rate functions 
and have been suggested as one approach to study the sublethal effects 
of pollutants on marine organisms (Bayne et al., 1979; Cole, 1979). In 
such studies, energy intake and expenditure are quantified so that the 
effects of the pollutants on energy accumulation for growth and 
reproduction (termed scope for growth) are determined. Results from 
bioenergetic analysis then provide clues on the mode of action of pollutants on the whole organism. For example, significant reduction 
in feeding rate as a result of hydrocarbon exposure has been documented 
for the bivalve Mytilus edulis (Widdows et al., 1982; Stickle et al., 
1985), the gastropod Thais lima (Stickle et al., 1984) and the seastar 
Evasterias troschelii (O'Clair and Rice, 1985). Changes in oxygen 
consumption in organisms exposed to crude oil were both dose- and 
species-dependent (see Edwards, 1978; Widdows et al., 1982; Stickle et 
al., 1984, 1985). Bioenergetic studies have also been useful as 
indicators of stress in natural populations of Mytilus edulis (Phelps 
et al., 1981).

Currently, other than short-term studies using larvae (Johns and 
Pechenik, 1980; Capuzzo and Lancaster, 1981), there is little 
information on the effects of crude oil exposure on the bioenergetics 
of crustacean species. In addition, there are few experimental data 
correlating changes in scope for growth with simultaneous growth or 
reproductive measurements. It is often assumed that changes in scope
for growth are accompanied by changes in growth and/or reproduction. In this study, we investigated the effects of exposure to the water-soluble fraction of South Louisiana crude oil on the bioenergetics of the blue crab, *Callinectes sapidus*, a commercially important species along the Atlantic and Gulf Coast. In addition, we correlated changes in bioenergetics with data on crab growth and molting. We also determined the LC50 of *C. sapidus* exposed to the WSF of crude oil over 21 days so that we could relate crude oil levels which cause long-term sublethal stress to the crab's long-term tolerance limits.

**MATERIALS AND METHODS**

**Experimental Design**

Juvenile *C. sapidus* and *Palaemonetes pugio* (serving as food) were collected from Ocean Springs, Mississippi, USA. Only intermolt crabs were used at the beginning of each experiment. Fifteen crabs (11.0-24.8 mm carapace width) per treatment were exposed to six aromatic hydrocarbon concentrations in order to determine their LC50 (predicted water-soluble aromatic hydrocarbon concentration that would kill 50% of the exposed individuals in a defined period). Crabs were fed abdominal tissue of freshly killed *P. pugio* twice daily. Mortality was monitored over a 21-d exposure period.

Effects of crude oil exposure on the bioenergetics of *C. sapidus* were determined from physiological rate measurements during a separate experiment using eight crabs per exposure concentration. The scope for growth (*P*) or energy incorporated into somatic growth and gamete production (Warren and Davis, 1967) is based on the balanced energy equation of Winberg (1960):

\[ C - F = Ab = R + U + P \]
where \( C \) = energy (food) consumed, \( F \) = energy lost as feces, \( Ab \) = energy absorbed from the food, \( R \) = energy respired, \( U \) = energy excreted as ammonia and \( P \) = production or scope for growth. \( P \) is not measured directly but is calculated by subtracting energy respired and excreted from energy absorbed from food consumed. Using Winberg's (1960) balanced energy equation, \( P \) or scope for growth = \( Ab - (R + U) \).

**Experimental Procedure**

A flow-through extraction apparatus described by Nunes and Benville (1978) produced the crude oil water-soluble fraction. The effluent was diluted with seawater to obtain water used at the highest dose. A second manifold provided oil-free seawater. Seawater of 20\(^{\circ}\)/oo salinity and 24\(^{\circ}\) C were used. Target aromatic hydrocarbon concentrations were obtained by adjusting the ratio of the water soluble effluent stock and seawater flowing into each of the dosing tanks. Aromatic hydrocarbon concentrations of 272 ± 12 (S.E.M.), 661 ± 36, 1,025 ± 41, 1,645 ± 48, 2,649 ± 78 and 4,831 ± 135 ppb were used to determine blue crab LC50 values. Hydrocarbon concentrations of 820 ± 45, 1,476 ± 52 and 2,504 ± 78 ppb were used to determine the effects of crude oil WSF on the bioenergetics of blue crabs. Each exposure tank had a flow rate of 120 ml·min\(^{-1}\) to maintain oxygen tensions above 80 mmHg and ammonia levels below 20 \( \mu \)M·l\(^{-1}\).

The aromatic hydrocarbon concentrations of seawater were monitored daily using ultraviolet spectrophotometry (Neff and Anderson, 1975) and the stock WSF concentrations were measured by gas chromatography. Seawater containing the water-soluble fraction of crude oil was extracted with hexane and its UV absorbance measured at 220 nm, the wavelength of maximum absorbance for naphthalenes. The concentrations of 18 aromatic hydrocarbons in the water-soluble fraction of crude oil,
extracted with methylene chloride, were analyzed using gas chromatography: toluene; ethylbenzene; o-, m- and p-xylene; n-propylbenzene; mesitylene; naphthalene; 1-, 2-methylnaphthalene; 2,6-dimethylnaphthalene; acenaphthylene; dibenzothiophene; anthracene; fluoranthene; pyrene; benzanthracene and benzopyrene. Total aromatic hydrocarbon concentration was then estimated from the sum of the concentrations of these 18 hydrocarbons; these include the most toxic components of the water-soluble fraction of crude oil (Rice et al., 1977).

For bioenergetic determinations, juvenile blue crabs (15.5-24.1 mm carapace width) were fed preweighed abdomen of freshly killed P. pugio twice daily in individual compartments. Uneaten shrimp was removed at the end of each feeding period, blotted dry and weighed. Food consumption was calculated by subtracting the weight of the uneaten food from the weight of the food provided at the beginning of each feeding period. Weight changes of uneaten food were found to be negligible. Wet weight of food consumed was converted to dry weight using the equation: \( \text{Dry wt} = \text{Wet wt} \cdot 0.2426 \pm 0.0046 \text{ SE, n} = 28 \). The mean energy content of shrimp abdominal tissue was determined using a Phillipson microbomb calorimeter and was \( 4.646 \pm 0.091 \text{ cal mg}^{-1} \) dry tissue \( \text{n=6} \). The food absorption efficiency of \( C. sapidus \) was determined by the Conover method (1966). Energy absorbed (Ab) per day was calculated by multiplying the ingested energy per day (C) by the absorption efficiency.

Respiration (R) of \( C. sapidus \) was determined using flow-through respirometry (Stickle et al., 1985). Oxygen consumption rates were converted to energy expenditure rates by using the oxycalorific value
of 4.8 calories per ml of oxygen consumed (Crisp, 1971).

Energy expenditure due to excretion (U) was determined by measuring ammonia excretion rate during respirometry. Ammonia levels were determined according to Grasshoff and Johanssen's (1972) modification of the phenol-hypochlorite method of Solorzano (1969). Energy equivalent of 0.0832 calories\(\mu\text{mol}^{-1}\) ammonia was used to convert ammonia excretion to energy excreted (Elliot and Davison, 1975).

Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System (SAS Institute; Cary, NC). Data on the tolerance of *C. sapidus* to crude oil were analyzed using probit analysis and presented as the LC50 + the 95% fiducial limits (Silverstone, 1957). Non-overlap of the 95% fiducial limits was used as criteria to determine significant differences between individual LC50's. All physiological rate functions were standardized to weight specific rates. Allometric equations relating rate functions to body size were not used since our primary consideration was to compare the various components of the crab energy budget rather than determining how rate functions change with body size. Scope for growth was calculated by integrating the various energy budget components of individuals rather than from means of treatment groups. Analysis of variance was used to analyze variation among treatments and Duncan's Multiple Range Test was used to identify significant differences between treatments.

RESULTS

Survival

*Callinectes sapidus* were very tolerant to the water soluble fraction (WSF) of South Louisiana crude oil. Survival was 100% at 1,645
± 48 ppb and lower. Although the predicted LC50 declined from 4,501 ± 833 ppb (+ 95% fiducial limits) on day 7 to 3,927 ± 778 ppb on day 21 (Fig. 1), the decline was not statistically significant.

Energy Intake

Rates of energy absorption (Ab), based on the feeding rate of C. sapidus on Palaemonetes pugio and absorption efficiency, varied inversely with exposure level and exposure time (Fig. 2). A significant difference in energy intake was observed at all times between crabs exposed to the WSF of crude oil and control crabs. The average energy intake of crabs exposed to 820 ± 45, 1,476 ± 52 and 2,504 ± 78 ppb over the 21-d experiment was 70.9, 59.5 and 48.8% of that of control crabs (Table 1; Fig. 3).

There were no significant differences in absorption efficiency as a function of petroleum hydrocarbon exposure. An average absorption efficiency of 89.6% was used to calculated the energy absorption rate (Ab) from the rate of energy intake (C).

Energy Expenditure

Total energy expenditure rates, the sum of energy expenditure due to respiration (R) and excretion (U), of crabs exposed to the highest concentration of WSF of crude oil (2504 ppb) were not significantly different than the control crabs (Fig. 3; Table 1). However, the total energy expenditure rates of crabs exposed to 2504 ppb aromatic hydrocarbon averaged over the 21-d experiment were significantly higher than those exposed to 820 and 1476 ppb. Respiratory and ammonia excretion rate determinations after 7, 14 and 21 days of exposure to aromatic hydrocarbons indicate, in general, higher energy expenditure rates in crabs exposed to 2504 ppb than control crabs or crabs exposed to 820 and 1476 ppb (Fig. 4). Control crabs exhibited a pattern of
Fig. 1. LC50 of *Callinectes sapidus* exposed to the WSF of South Louisiana crude oil as a function of exposure time. (Mean ± 95% confidence limits)
Fig. 2. Weight specific energy absorption rates of Callinectes sapidus as a function of total aromatic hydrocarbon exposure concentration and exposure time. (cal'g wet⁻¹ day⁻¹) (Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different)
Table 1. The effects of the WSF of South Louisiana crude oil on the bioenergetics of *Callinectes sapidus* averaged over 21 days. (Energetic components expressed as cal g wet\(^{-1}\) day\(^{-1}\)) Letters represent Duncan's multiple range test for difference between doses; means with the same letters are not significantly different.
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<th>1476</th>
<th>2504</th>
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<td>Energy Ingested (C)</td>
<td>197.9</td>
<td>140.4</td>
<td>117.7</td>
<td>96.6</td>
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<tr>
<td>Energy Absorbed (Ab)</td>
<td>177.3</td>
<td>125.8</td>
<td>105.5</td>
<td>86.6</td>
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<tr>
<td>Energy Respired (R)</td>
<td>38.5</td>
<td>34.3</td>
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<td>42.0</td>
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<td>Energy Excreted (U)</td>
<td>3.7</td>
<td>2.2</td>
<td>2.0</td>
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<td>Total Energy Expenditure</td>
<td>42.2</td>
<td>36.6</td>
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<td>46.3</td>
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<td>% Due to Respiration</td>
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<td>93.7</td>
<td>95.1</td>
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<td>6.3</td>
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<td>9.0</td>
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<td>Scope for Growth (P)</td>
<td>135.1</td>
<td>89.2</td>
<td>68.1</td>
<td>40.4</td>
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<td>Gross Growth Efficiency</td>
<td>66.5 A</td>
<td>60.8 AB</td>
<td>53.7 B</td>
<td>39.0 C</td>
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<tr>
<td>Net Growth Efficiency</td>
<td>74.2 A</td>
<td>67.8 AB</td>
<td>59.9 B</td>
<td>43.5 C</td>
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Fig. 3. Weight specific scope for growth of *Callinectes sapidus* and its components (P = Ab - (R + U)) as a function of total aromatic hydrocarbon concentration averaged over 21 days. (A = Energy absorption rates; B = Total energy expenditure rates; C = Scope for growth) Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different.
Fig. 4. Weight specific total energy expenditure rates of *Callinectes sapidus* as a function of total aromatic hydrocarbon exposure concentration and exposure time. (cal g wet$^{-1}$ day$^{-1}$) Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different.
continuous decrease in total energy expenditure rates over time which was not evident in crabs exposed to the WSF of South Louisiana crude oil. This was due at least in part to not using the allometric function relating total energy expenditure to body weight. The slopes (b) for crabs exposed to 0, 820, 1476 and 2504 ppb were 0.78, 1.02, 1.30 and 0.94, respectively.

Energy expenditure due to respiration (R) accounted for most of the energy expenditure, varying between 88.1 and 96.0%. Respiratory energy expenditure, as a percentage of the total energy expenditure, was significantly higher over the 21-d experiment in crabs exposed to 820 and 1476 ppb aromatic hydrocarbons than control crabs or crabs exposed to 2504 ppb aromatic hydrocarbons (Table 1). The reverse was true for percentage energy expenditure due to ammonia excretion (U).

Scope for Growth

The scope for growth (P) of *C. sapidus* varied inversely with aromatic hydrocarbon concentration and time over the 21-d experimental period (Fig. 5). The scope for growth of control crabs was significantly higher than that of crabs exposed to the WSF of crude oil in all cases. Significant differences in scope for growth were found between crabs exposed to 820, 1476 and 2504 ppb on day 7 but not on days 14 and 21. Averaged over 21 days, the scope for growth of crabs exposed to 820 and 1476 ppb petroleum hydrocarbons was significantly higher than that of crabs exposed to 2504 ppb (Table 1). The scope for growth of crabs exposed to 820, 1476 and 2504 ppb was 66, 50 and 30% of that of control crabs over the 21-d experimental period. Fig. 3 indicates that the rate of energy intake is the primary determinant of scope for growth decreases in crabs exposed to crude oil.
Fig. 5. Weight specific scope for growth of *Callinectes sapidus* as a function of total aromatic hydrocarbon exposure concentration and exposure time. (cal.g wet\(^{-1}\) *day\(^{-1}\)) Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different.
Growth and Molting

Exposure to the WSF of South Louisiana crude oil inhibited growth and molting of *C. sapidus* (Fig 6). Molting occurred twice in 30 of the 32 crabs examined during the study. Growth, measured as percent size (carapace width) increase after molting, was significantly reduced in crabs exposed to 1476 and 2504 ppb aromatic hydrocarbons with the first molt and was significantly reduced at all hydrocarbon concentrations with the second molt. Growth during the second molt was inversely related to crude oil levels, decreasing from 29.2% in control crabs to 16.9% in crabs exposed to 2504 ppb aromatic hydrocarbons. The intermolt period, measured as the number of days between ecdysis, was prolonged in crabs exposed to crude oil and was positively correlated to the exposure concentration. The intermolt period was 11.4 days in control crabs and 15.7 days in crabs exposed to 2504 ppb aromatic hydrocarbons.

We found highly significant correlation between changes in scope for growth and changes in observed growth in *C. sapidus* as a result of exposure to the WSF of crude oil for 21 days (see Table 2). Growth, in terms of size, can be related to scope for growth by:

\[
\% \text{ Size Increase} = 27.8577 + 0.2710 \times \text{Scope for Growth} \quad r^2=0.8416
\]

Growth, in terms of wet weight, can be related to scope for growth by:

\[
\% \text{ Wt Increase} = 100.3172 + 2.0228 \times \text{Scope for Growth} \quad r^2=0.9237
\]

**DISCUSSION**

*Callinectes sapidus* juveniles were extremely tolerant to long-term petroleum aromatic hydrocarbon exposure. One hundred percent survival over 21 days was observed up to 1,645 ± 48 ppb. The 21-day LC50 for *C. sapidus* (3,927 ± 778 ppb) is higher than that observed for *Mytilus edulis* (1930 ppb) (Stickle et al., 1985), *Thais lima* (961 ppb) (Stickle
Fig. 6. Effects of exposure to the WSF of South Louisiana crude oil on the intermolt period and size increase per molt of Callinectes sapidus. (Mean ± S.E.M.; • = Number of days between ecdysis; □ = % size increase after the first molt; ▲ = % size increase after the second molt)
INTERMOLT PERIOD (DAYS)

TOTAL AROMATIC HYDROCARBONS (PPB)

% CARAPACE WIDTH INCREASE

INTERMOLT PERIOD (DAYS)

TOTAL AROMATIC HYDROCARBONS (PPB)

% CARAPACE WIDTH INCREASE
Table 2. Scope of growth and observed growth of *Callinectes sapidus* exposed to the WSF of South Louisiana crude oil summarized over 21 days. (means ± SEM; n=8)
<table>
<thead>
<tr>
<th>Aromatic Hydrocarbon Concentration (ppb)</th>
<th>0</th>
<th>820</th>
<th>1476</th>
<th>2504</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scop for Growth (cal)</td>
<td>135</td>
<td>89</td>
<td>68</td>
<td>40</td>
</tr>
<tr>
<td>Carapace Width (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>18.22±0.59</td>
<td>17.84±0.62</td>
<td>17.88±0.55</td>
<td>19.01±0.92</td>
</tr>
<tr>
<td>Final</td>
<td>29.65±1.10</td>
<td>28.39±1.21</td>
<td>25.67±1.01</td>
<td>25.92±0.80</td>
</tr>
<tr>
<td>% Increase</td>
<td>62.60±2.32</td>
<td>58.98±2.49</td>
<td>43.52±3.27</td>
<td>37.26±2.99</td>
</tr>
<tr>
<td>Wet Weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>0.50±0.05</td>
<td>0.45±0.05</td>
<td>0.44±0.03</td>
<td>0.52±0.09</td>
</tr>
<tr>
<td>Final</td>
<td>2.23±0.19</td>
<td>1.90±0.24</td>
<td>1.44±0.13</td>
<td>1.31±0.10</td>
</tr>
<tr>
<td>% Increase</td>
<td>354.0±20.4</td>
<td>315.1±13.6</td>
<td>228.3±17.9</td>
<td>171.2±16.8</td>
</tr>
</tbody>
</table>
et al., 1984) and Pandalus borealis (100 ppb) (Stickle et al., 1986). Although the temperature, salinity and crude oil used were different in the present study, our results clearly indicate that C. sapidus is one of the most tolerant species tested so far to long-term petroleum hydrocarbon exposure. This is consistent with previous reports concerning the tolerance of C. sapidus to other types of stress, such as temperature and salinity extremes, hypoxia (reviewed by Williams, 1974) and starvation (Wang and Stickle, 1986).

The major component of the energy budget of C. sapidus affected by petroleum hydrocarbon exposure was food consumption. The consumption rate declined in a linear manner with increasing exposure concentration. This trend confirms the findings of other investigators concerning the influence of environmental stressors on feeding. Reduced feeding, as a result of exposure to petroleum aromatic hydrocarbons, has been demonstrated in the bivalve Mytilus edulis (e.g. Widdows et al., 1982; Stickle et al., 1985), the gastropod Thais lima (Stickle et al., 1984) and larvae of the crustacean Cancer irroratus (Johns and Pechenik, 1980). In addition, Stickle (1985) has shown that variation in ingestion rate is the primary determinant of scope for growth along environmental factor gradients of salinity and temperature in four species of carnivorous marine invertebrates.

The reduction in food consumption in crabs exposed to crude oil may reflect a narcotizing effect of the oil on sensory functions (Johns and Miller, 1982). Although the feeding rate in Cancer irroratus larvae was unaffected by copper and cadmium exposure it was reduced when exposed to sublethal concentrations of crude oil (Johns and Pechenik, 1980). Derby and Atema (1981) and Pearson et al. (1981) have demonstrated impairment in the ability of decapod crustaceans to detect
food cues during exposure to drilling mud and petroleum hydrocarbons, respectively. Difficulty in food localization was observed in the present study in crabs exposed to the two higher WSF levels (1,476 and 2,504 ppb) as evidenced by their delayed response and prolonged "groping" or search behavior once food was provided. In view of the importance of energy intake in an organism's energy budget, further studies in such areas as chemosensory, food palatability and feeding motivation are needed in order to enhance our understanding of the mechanisms regulating energy intake in stressed organisms.

Total energy expenditure (R+U), in contrast to energy consumption, did not vary with hydrocarbon exposure concentration in a linear manner. Energy expenditure was greatest in crabs exposed to the highest dose (2,504 ppb aromatic hydrocarbons). Their average energy expenditure rate over 21 days was not significantly different from that of control crabs but was significantly higher than that of crabs exposed to 1476 and 820 ppb. This general pattern was true for both energy expenditure due to respiration and excretion. The physiological mechanisms responsible for such a pattern of energy expenditure is unclear. Activity may have played a role and may be a behavioral adaptation to stress in general. Mobile species may respond to low level stress by becoming quiescent, waiting for improved conditions while high level stress may induce an escape behavior. If activity pattern is influenced by environmental stress in such a manner in blue crabs, it would explain in part the pattern of energy expenditure observed in the present study. Other investigators have also reported pollutant concentration dependent patterns in oxygen consumption rates of crustaceans. Smith and Hargreaves (1984) reported depressed oxygen
consumption rate in \textit{Neomysis americana} exposed to 0.1 mg\textsuperscript{-1} but elevated rates in mysids exposed to 1.1 mg\textsuperscript{-1} naphthalene. Percy (1977) also reported such a pattern of oxygen consumption for an arctic amphipod exposed to crude oil. However, patterns of respiratory and ammonia excretion are not always uniform. For example, Laughlin and Neff (1979b, 1980) reported a general increase in oxygen consumption in larval and juvenile \textit{Rhithropanopeus harrisii} exposed to phenanthrene. Carr and Lindin (1984) recorded increased rates of oxygen consumption and ammonia excretion in \textit{Gammarus salinus} exposed to North Sea crude oil for 10 days. Reduced oxygen consumption, however, was found in \textit{Crangon crangon} and larval \textit{Homarus americanus} exposed to sublethal concentrations of crude oil (Edwards, 1978; Capuzzo \textit{et al.}, 1984).

Clearly, the effects of petroleum hydrocarbons on metabolic maintenance are dependent upon the combined effects of a number of factors, such as temperature, salinity and composition of the pollutant in question. In addition, a factor often overlooked is the concentration of the pollutant used relative to the long-term tolerance limit of the species, as recently discussed by Stickle \textit{et al.} (1984). Thus, investigations on the long-term tolerance of species to pollutant levels that are environmentally realistic should be encouraged.

The scope for growth (P) varied inversely with the concentration of petroleum aromatic hydrocarbons. Similar results have been demonstrated for bivalves and gastropods (Widdows \textit{et al.}, 1982; Stickle \textit{et al.}, 1984, 1985). It is interesting to note that while energy intake decreased with increased oil level, the pattern of energy expenditure differs between molluscs and the present crustacean species. While bivalves and gastropods decreased energy expenditure at high aromatic hydrocarbon concentrations (Gilfillan, 1975; Stickle \textit{et al.}}
al., 1984), C. sapidus expended the greatest amount of energy at high aromatic hydrocarbon concentrations. The result is a much more pronounced decrease in scope for growth at increased hydrocarbon concentrations. Such differences may have a phylogenetic basis. Fig. 3 indicates that the scope for growth of C. sapidus may become negative at a WSF aromatic hydrocarbon concentration near the 21-d LC50.

Our results indicate that both the duration of exposure to crude oil and exposure level were important in reducing growth and molting in juvenile C. sapidus. The reduction in growth was more pronounced with the second molt than with the first (see Fig. 6), reflecting perhaps a cumulative effect during long-term exposure to crude oil. Delayed development, reduced growth and delayed molting as a result of exposure to petroleum hydrocarbons have been reported for many species of crustacean larvae (e.g. Wells, 1972; Caldwell et al., 1977; Mecklenburg et al., 1977; Capuzzo and Lancaster, 1982). Responses among post metamorphic species exposed to petroleum hydrocarbons were not consistent. Cantelmo et al. (1981, 1982) found decreased growth and delayed molting in juvenile C. sapidus exposed to benzene and dimethylnaphthalene. Growth and reproduction were reduced and molting was impaired in Daphnia pulex when exposed to phenanthrene (Geiger and Buikema, 1982). Laughlin et al. (1978) however, did not find a significant difference in growth rate after 6 months between control Rhithropanopeus harrisii and crabs exposed to the WSF of No. 2 fuel oil. Similar conclusions were reached for Homarus americanus exposed to the WSF of crude oil for 30 days (Wells and Sprague, 1976) and 78 days (Capuzzo and Lancaster, 1981). Cucci and Epifanio (1979) did find significant delays in molting in Eurypanopeus depressus through the
early adult stages when exposure to the WSF of crude oil commenced with the first zoeal stage. Growth rates were reduced in *Crangon crangon* (Edwards, 1978) and molting success decreased in juvenile *Chionoecetes bairdi* (Karinen and Rice, 1974) when exposed to crude oil WSF.

We found evidence for a causal mechanism underlying the observed reduction in growth and delayed molting in *C. sapidus* exposed continuously to the WSF of South Louisiana crude oil for 21 days. Scope for growth or energy retained for somatic growth and reproduction was greatly reduced in crabs exposed to crude oil WSF as a result of reduced energy intake without a concomitant reduction in maintenance costs. This pattern parallels the reduced growth and delayed molting observed with increasing crude oil level.
CHAPTER TWO:
BIOENERGETICS AND THE USE OF RNA:DNA AND PROTEIN:DNA RATIOS AS
INDICATORS OF STRESS IN THE BLUE CRAB, CALLINECTES SAPIDUS,
DURING EXPOSURE TO CRUDE OIL

INTRODUCTION

Changes in growth, which integrate all physiological processes, often accompany environmental stress. The mechanism by which growth is affected however, is often unclear because of the generalized nature of organismal response to stress (Selye, 1951). Bioenergetic studies have been one approach to the study of sublethal effects of pollutants on organisms. In such studies, feeding, absorption efficiency, respiration and excretion rates of animals exposed to pollutants are measured and compared to those of control animals so that the physiological processes affected may be quantified. All rate measurements are then converted to a common energy unit and summed so that a net "scope for growth" or energy accumulated for growth and reproduction may be calculated. Widdows et al. (1982) Stickle et al. (1984, 1985, 1986) and Wang and Stickle (1986a) have used this approach to quantify changes in the scope for growth of marine organisms as a result of exposure to petroleum hydrocarbons.

Johns and Pechenik (1980), O'Clair and Rice (1985) and Stickle (1985) among others have shown that feeding is often affected in stressed organisms. In addition, Edwards (1978), Cantelmo (1982), Capuzzo et al. (1984) and Stickle and Bayne (submitted) have reported that growth is affected also. Since changes in nucleic acid content and ratios have been used as reliable and sensitive indicators of nutritional stress (Buckley, 1980; Wang and Stickle, 1986b) and growth of organisms (Bulow et al., 1978; Dortch et al., 1983), it may be
possible to use them as indicators of stress in organisms exposed to pollutant stress.

Sutcliffe (1965) first proposed the use of nucleic acid determinations to estimate growth rates in planktonic organisms. The rationale is conceptually straightforward. Ribonucleic acid (RNA) is produced during gene transcription and is a necessary step for protein synthesis. Changes in RNA content would theoretically mirror cellular biosynthetic activity. Deoxyribonucleic acid (DNA) is the basis of organismal molecular identity and is usually constant per cell (Leslie, 1955). The ratio of RNA to DNA would thus provide information about the physiological state of an organism. Protein is produced during gene translation and is dependent on RNA. The quantity of protein produced should be correlated to the level of RNA present. Thus the ratio of protein to DNA could also provide information about the growth of an organism. Organisms in good condition and growing are expected to have high ratios of RNA to DNA and protein to DNA relative to those that are stressed.

In this study, we determined changes in growth and bioenergetics of the blue crab, *Callinectes sapidus*, exposed to the water-soluble fraction of South Louisiana crude oil in the laboratory. In addition, we analyzed crab nucleic acid and protein content and correlated the ratios of RNA to DNA and protein to DNA to measured growth and scope for growth. Our objectives are to determine how pollutant stress affects growth, in terms of weight and size, possible mechanisms for such effects and whether ratios of RNA to DNA and protein to DNA can serve as instantaneous indicators of environmental stress.
MATERIALS AND METHODS

Experimental Design

Juvenile blue crabs were exposed to the water soluble fraction of South Louisiana crude oil in the laboratory for 21 days. Respiratory and ammonia excretion rates were determined weekly while feeding rates were determined daily. Feeding rates were multiplied by the absorption efficiency to calculate food absorbed. These rates were compared to those of control crabs to determine the effects of exposure to petroleum hydrocarbons on the bioenergetics of blue crabs. All rate functions were transformed to caloric equivalents. The balanced energy equation of Winberg (1960):

\[ C - F = Ab = R + U + P \]

was used to integrate the rates to calculate crab scope for growth. C = energy (food) consumed, F = energy lost as feces, Ab = energy absorbed from the food, R = energy respired, U = energy excreted as ammonia and P = production or scope for growth. P was not measured directly but calculated by subtracting energy respired and excreted from food energy absorbed (P = Ab - R - U).

Crabs were frozen in liquid nitrogen after the 21 day exposure period and stored at -70°C for biochemical analyses. Additional crabs, exposed to aromatic hydrocarbons concurrently, were sampled on days 7 and 14 for biochemical analyses.

Experimental Procedures

Crabs were exposed in individual compartments to the water-soluble fraction of crude oil in a flow-through system. The mean initial ash-free dry weight and carapace width of the crabs was 104.6 ± 8.1 (S.E.) mg and 18.2 ± 0.3 mm respectively. Values of 73% water and 39% ash (percent of dry tissue) were used to calculate the initial ash-free dry
weight of blue crabs. These values were those of crabs collected from the field during collection of experimental crabs. Petroleum hydrocarbon concentrations of $0, 820 \pm 45, 1,476 \pm 52$ and $2,504 \pm 78$ ppb aromatic hydrocarbons were used. The flow-through system and procedures used to determine and maintain stable hydrocarbon concentrations have been described in detail previously (Wang and Stickle, 1986a).

Crabs were fed preweighed abdomen of freshly killed grass shrimp, *Palaemonetes pugio*, twice daily. Uneaten shrimp were removed at the end of each feeding period, blotted dry and weighed. Food consumed was the difference in weight between shrimp provided and removed. Weight changes of food uneaten during a 12 hour feeding period could not be detected. Dry weight of food consumed was obtained by multiplying the wet weight by $0.2426$ ($n = 28$, S.E. = 0.0046), the decimal fraction of shrimp wet wt that was dry tissue. The mean energy content of shrimp abdominal tissue was determined using a Phillipson microbomb calorimeter and was $4.65 \pm 0.09 \text{ cal} \cdot \text{mg}^{-1}$ dry tissue ($n = 6$). Absorption efficiency was calculated by the Conover method (1966) using crab feces collected daily. Energy absorbed (Ab) was calculated by multiplying the ingested energy per day (C) by the absorption efficiency.

Crab respiration (R) was determined using flow-through respirometry (Stickle et al., 1985) and converted to energy respired using the oxycalorific value of 4.8 calories per ml oxygen consumed (Crisp, 1971). Ammonia excretion was determined using water flowing past crabs during respirometry experiments. Ammonia levels were determined according to Grasshoff and Johanassen's (1972) modification of the phenol-hypochlorite method of Solorzano (1969). Energy excreted
(U) was obtained from the ammonia excretion rate using the conversion factor 0.0832 calories·µM⁻¹ ammonia excreted (Elliot and Davison, 1975).

DNA and RNA levels were determined colorimetrically. Individual frozen crabs were pulverized in a mortar and pestle (cooled in liquid nitrogen) and homogenized in buffer (100 mM NaCl, 50 mM Tris, 10 mM MgCl₂ and 1 mM EDTA) with a Tekmar Tissuemizer. An aliquot of the homogenate was removed for protein analysis before an equal volume of 20% TCA was added. After 20 min in an ice bath, the samples were centrifuged in a Microfuge at 12,000 x g for 5 min. The resulting pellet was analyzed for nucleic acid content. Diphenylamine (modified procedure of Burton, 1956) for DNA and orcinol (modified procedure of Ceriotti, 1955) for RNA, respectively, were used as the chromogenic substance, as outlined by Webb and Levy (1958). Standards were prepared from calf thymus DNA and yeast RNA according to the procedure of Shatkin (1969) to construct standard curves. Since orcinol also reacts with DNA, optical absorbance of orcinol reactions was corrected for DNA interference using a orcinol-DNA standard curve.

Protein concentrations were determined using Bradford's (1976) Coomassie blue dye binding assay as outlined by Peterson (1983). Bovine serum albumin was used to construct the standard curve. Assays for DNA, RNA and protein were done in duplicate and all chemicals were obtained from Sigma Chemical Co.

An aliquot of the pulverized crab described above was dried at 70° for 24 h and then ashed in a muffle furnace at 450° for 12 h to determine percentage dry weight and ash, respectively.
Statistical Analysis

Results were standardized to crabs of equal weight according to Neter and Wasserman 1974 in order to analyze the effects of exposure to petroleum hydrocarbons independent of any differences in crab weight. Regression equations, either \( Y = aW^b \) or \( Y = a + bW \), were used to model the data, where \( Y \) is the physiological or biochemical measurement, \( W \) = crab weight and \( a \) and \( b \) are the intercept and slope of the regression, respectively. \( Y' \), the adjusted \( Y \), was calculated using the equation: \( Y' = Y - b(W - \bar{W}) \), where \( \bar{W} \) is mean weight of all crabs used. Analysis of covariance was used to test for differences in the regression slope \( b \) between treatments. Separate regression slopes were used if they were significantly different from each other. Statistical modeling was performed using the General Linear Models procedure (SAS Institute, Cary, North Carolina). Duncans multiple range test was used to test for significant differences between treatment means.

RESULTS

Exposure to aromatic hydrocarbons had a significant effect on the bioenergetics of C. sapidus. Rates of energy absorption were significantly reduced as a result of exposure to the water-soluble fraction of crude oil (Fig 7a). Absorption efficiency did not vary as a function of exposure concentration and an averaged value of 89.6% was used to calculate energy absorption rates (\( Ab \)) from rates of energy consumption (\( C \)). Since absorption efficiency did not differ as a function of hydrocarbon exposure concentration, the reduced rates of energy absorption among crabs exposed to the water-soluble fraction of crude oil were due entirely to reduced food consumption. Energy expenditure rates due to respiration and ammonia excretion (\( R + U \)) were
highest for crabs exposed to 2504 ppb (Fig. 7b). The rate for crabs exposed to 2504 ppb were not significantly different from that of control crabs but was significantly higher than that of crabs exposed to 820 and 1476 ppb. Crabs exposed to aromatic hydrocarbons had significantly reduced scope for growth (P) (Fig. 7c). Scope for growth was inversely related to the petroleum hydrocarbon exposure concentration.

The O:N ratio (molar ratio of oxygen consumed to nitrogen excreted) of C. sapidus did not vary significantly as a function of hydrocarbon exposure concentration (p > 0.06) and the average value was 22.3. Using the nitrogen quotient (NQ) as an indicator of metabolic substrate used (Gnaiger, 1983) (NQ = 2 / O:N ratio), an averaged value of 0.09 indicates that protein as well as non-protein metabolic substrates were catabolized.

Crab growth, in terms of both size and ash-free dry weight, was inversely related to the concentration of petroleum hydrocarbons (Fig 8a). The number of crabs molting during the 21 d exposure period did not appear to be affected as 30 of the 32 crabs molted twice. Molting was delayed however, in crabs exposed to hydrocarbons of 1476 and 2511 ppb aromatic hydrocarbons since the intermolt period was significantly longer in these crabs than control crabs and crabs exposed to 820 ppb (Fig. 8b).

Crab RNA concentration was inversely related to hydrocarbon concentration on day 7 with RNA concentration of crabs exposed to 2504 ppb significantly lower than that of control crabs (Table 3). By day 14, crabs exposed to crude oil had significantly lower concentrations of DNA, RNA and protein. Such differences however, were not observed on day 21, suggesting a restoration of equilibrium in biochemical
Fig. 7. Weight specific scope for growth of *Callinectes sapidus* and its components \((P = Ab - (R + U))\) averaged over 21 d as a function of total aromatic hydrocarbon concentration. a). Energy absorbed; b). Total energy expenditure; c). Scope for growth. All rate were adjusted to crabs of 255 mg ash free dry wt, the average weight of all crabs used. Letters represent Duncan's multiple range test for differences between doses; means with the same letters are not significantly different.
CALORIES - G - ASH-FREE DRY WT-1*DAY-1

Ab

R+U

TOTAL AROMATIC HYDROCARBONS(ppb)
Fig. 8. Effects of exposure to the water-soluble fraction of crude oil on growth and molting of *Callinectes sapidus*. a). Percent growth in ash free dry wt and carapace width over 21 d of exposure; b). Intermolt period. Letters represent Duncan's multiple range test for differences between doses; means with the same letter are not significantly different.
INTEL MOLT PERIOD PERCENT GROWTH

ASH-FREE ORY WEIGHT CARAPACE WIDTH

INTERMOLT PERIOD (days)

TOTAL AROMATIC HYDROCARBONS (ppb)

21 DAY LC50
Table 3. DNA, RNA and protein concentration of *Callinectes sapidus* exposed to the WSF of South Louisiana crude oil. Concentrations were adjusted to crabs of 255 mg ash-free dry wt. (ug·mg⁻¹ ash-free dry wt ± SEM; n = 8) Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different.
<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>820</th>
<th>1476</th>
<th>2504</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 7</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DNA</td>
<td>2.17±0.18A</td>
<td>2.33±0.20A</td>
<td>2.25±0.32A</td>
<td>2.44±0.24A</td>
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<tr>
<td>RNA</td>
<td>11.42±0.55A</td>
<td>9.73±1.03A</td>
<td>9.12±0.77A</td>
<td>7.88±0.55B</td>
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<tr>
<td>Protein</td>
<td>365.83±21.67A</td>
<td>369.84±24.52A</td>
<td>377.30±12.76A</td>
<td>380.99±15.47A</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>2.47±0.14A</td>
<td>1.83±0.25B</td>
<td>1.80±0.07B</td>
<td>2.08±0.15AB</td>
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<td>RNA</td>
<td>12.32±1.38A</td>
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<tr>
<td>Protein</td>
<td>468.30±51.39A</td>
<td>381.98±15.08B</td>
<td>340.93±8.07B</td>
<td>355.63±8.99B</td>
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<tr>
<td><strong>Day 21</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DNA</td>
<td>2.11±0.38A</td>
<td>2.15±0.21A</td>
<td>2.70±0.33A</td>
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<tr>
<td>RNA</td>
<td>11.50±0.94A</td>
<td>6.98±0.67B</td>
<td>10.02±1.16A</td>
<td>9.92±0.65A</td>
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<tr>
<td>Protein</td>
<td>400.14±37.29A</td>
<td>431.55±19.28A</td>
<td>369.78±34.38A</td>
<td>418.93±17.35A</td>
</tr>
</tbody>
</table>
The RNA:DNA ratio of crabs exposed to 2504 ppb petroleum hydrocarbons was significantly lower than that of control crabs on day 7 (Fig. 9). By day 21, the RNA:DNA ratios were inversely related to the hydrocarbon exposure concentration. The RNA:DNA ratio of crabs exposed to 820, 1476 and 2504 ppb were 66, 65 and 54% of that of control crabs, respectively. Significant differences in protein:DNA ratios as a result of crude oil exposure were not observed until day 21 when the ratio of crabs exposed to 1476 and 2504 ppb were significantly lower than that of control crabs (Fig. 10). Protein:DNA ratios were positively correlated to RNA:DNA ratios with the correlation being highly significant (Fig. 11).

Ratios of RNA:DNA and protein:DNA were both positively correlated to crab scope for growth (Fig. 12) and measured growth in ash free dry wt (Fig. 13b, c). Crab scope for growth was also positively correlated to the measured growth of crabs (Fig. 13a). Although all regressions were significant, the fit (as indicated by the coefficient of determination, r²) was much better for the regression between actual growth and scope for growth.

DISCUSSION

Exposure to petroleum aromatic hydrocarbons significantly lowered the growth rate and scope for growth of juvenile C. sapidus. Growth, the result of integrated bioenergetic functions, is the mechanism by which the effect of environmental disturbance is passed on to the population and community level. Decreased growth (Tatem, 1977; Edwards, 1978; Cantelmo et al., 1982) and delayed molting (Cucci and Epifanio, 1979; Laughlin and Neff, 1979; Capuzzo and Lancaster, 1982) appears to be a general stress response among the crustaceans exposed
Fig. 9. RNA to DNA ratios of *Callinectes sapidus* exposed to the water-soluble fraction of crude oil for 21 d. Letters represent Duncan's multiple range test for differences between doses with day; means with the same letter are not significantly different. (n = 8 on day 7 and 7 on days 14 and 21)
TOTAL AROMATIC HYDROCARBONS (ppb)
Fig. 10. Protein to DNA ratios of *Callinectes sapidus* exposed to the water-soluble fraction of crude oil for 21 d. Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different. (n = 8 on day 7 and 7 on days 14 and 21)
TOTAL AROMATIC HYDROCARBONS (ppb)
Fig. 11. Correlation between ratios of protein to DNA and RNA to DNA of Callinectes sapidus exposed to the water-soluble fraction of crude oil for 21 d. (n = 32)
PROTEIN: DNA RATIO

Y = 43.27 + 32.7081 x

r² = 0.7584  p = 0.0001
Fig. 12. Correlations between the ratios of RNA:DNA and protein:DNA of *Callinectes sapidus* exposed to the water-soluble fraction of crude oil for 21 d and scope for growth averaged over 21 d. (n = 32)
PROTEIN: DNA RATIO

RNA: DNA RATIO

Y = 2.07 + 0.0060 X

r² = 0.3984 p = 0.0002

Y = 110.84 + 0.1940 X

r² = 0.3002 p = 0.0021

SCOPE FOR GROWTH (cal-g-ash-free dry wt/day)

0 200 400 600 800 1000

0 200 400 600 800 1000

0 2 4 6 8 10 12
Fig. 13. Correlations between indicators of stress and actual growth of *Callinectes sapidus* exposed to the water-soluble fraction of crude oil for 21 d. a). Scope for growth; b). Ratios of RNA to DNA; c). Ratios Protein to DNA.
PROTEIN: DNA RATIO

RNA:DNA RATIO

Y = 156.26 + 1.1594 x
r^2 = 0.7435 p = 0.0001

Y = 2.86 + 0.0075 x
r^2 = 0.3485 p = 0.0008

Y = 142.18 + 0.2203 x
r^2 = 0.2141 p = 0.0115

SCOPE FOR GROWTH (mg - ash-free dry wt)

GROWTH-ASH-FREE DRY WT (mg)
to sublethal levels of petroleum hydrocarbons. The positive correlation between ash-free tissue growth and scope for growth over 21 days was highly significant, indicating a bioenergetic basis for the reduction in growth among crabs exposed to petroleum hydrocarbons (Fig. 13a). However, the mechanism underlying the delay in molting among crabs exposed to 1476 and 2504 ppb is unclear. Because a significant inverse relation between the intermolt period and averaged daily scope for growth was found (days between molt = \(-0.0106 \text{ cal}^\prime \text{g} \text{ ash-free dry wt}^{-1} \cdot \text{day}^{-1} + 18.02; r^2 = 0.416; p < 0.001\)), we considered the possibility that molting was delayed in crabs exposed to petroleum hydrocarbons in order to compensate for decrease in scope for growth, i.e., molting was delayed until a similar amount of energy had been accumulated. This however, was clearly not the case. The amount of energy accumulated for growth during the intermolt period (daily scope for growth \(\times\) intermolt period), was significantly higher in control crabs than crabs exposed to crude oil (\(p < 0.001\)). Thus, although growth in tissue appears to have a bioenergetic basis, molting frequency does not.

It is interesting to note the difference in growth in terms of ash-free dry weight versus carapace width, i.e., the approximately seven fold difference in weight gained between control crabs and those exposed to 2511 ppb versus the 66% difference in terms of carapace width. This is perhaps expected since weight gained is a cubic function (i.e., how much of the shell is filled with tissue) while width increase is a linear function. However, in view of the large decrease in scope for growth and small gain in tissue after molting in crabs exposed to high hydrocarbon concentrations, the perplexing question becomes why do they molt at all? Molting results not only in
the loss of energy in the exuvia but also requires energy for the secretion and mineralization of a new cuticle, energy that could be devoted to tissue growth. We suggest that because of the highly aggressive nature of blue crabs, molting in order to increase in size as an anti-predation measure is of sufficient importance to take place even under stressful environmental conditions and reduced scope for growth.

Because physiological rate functions used to calculate scope for growth provide indications of the mechanisms underlying differences in growth, they clearly suggest that the reduced growth in C. sapidus was due primarily to reduced energy absorbed. Similar observations have been made by Stickle et al. (1986) for the prawn Pandalus borealis exposed to the water-soluble fraction of Cook Inlet crude oil. Blumer (1969) initially suggested that exposure to petroleum hydrocarbons could interfere with chemoreception and thus alter behavioral patterns. Subsequently, Pearson and Olla (1979) and Pearson et al. (1980) have shown that C. sapidus and Cancer magister, respectively, could detect aromatic hydrocarbons at concentrations of approximately 120 ppb. In addition, many investigators, including Atema and Stein (1974), Atema et al. (1979), Berman and Heinle (1980) and Busdosh (1981), have demonstrated altered feeding behavior in crustaceans as a result of exposure to petroleum hydrocarbons. It is still unclear, however, whether such altered feeding behavior is due to impairment in their ability to detect food cues during exposure (Derby and Atema, 1981; Pearson et al., 1981) or other causes such as altered food palatability and feeding motivation.

Energy expenditure rates (R + U) of crabs exposed to the highest
concentration (2511 ppb), although not significantly higher than those of control crabs, was significantly higher than those of crabs exposed to the lower concentrations (820 and 1476 ppb). This was true both for energy expenditure due to respiration and ammonia excretion. This general pattern of exposure concentration dependent respiratory rate have been documented for many species of crustaceans including *Palaemonetes pugio* and *Penaeus aztecus* (Anderson et al.), *Onisimus affinis* (Percy, 1977), *Lucifer faxoni* (Lee et al., 1978) and *Neomysis americana* (Smith and Hargreaves, 1984). Decreased energy expenditure rates of *C. sapidus* at low hydrocarbon concentrations may be due to decreased activity while elevated rate at high concentration may be due to stimulation of basal metabolism as Percy (1977) has shown for the amphipod, *Onisimus affinis*. This suggestion is supported by the findings of Anderson (1977) who correlated tissue hydrocarbon concentration in mysids and shrimp with respiratory rates. It is also possible however, that high respiratory rates at high exposure concentrations may also be a consequence of escape behavior among these highly mobile crustaceans.

The relationship between scope for growth and measured growth of *C. sapidus* was highly significant. Reduced growth in crabs exposed to crude oil resulted primarily from reduced energy intake. This effect was compounded by higher maintenance expenditures at increased hydrocarbon concentrations. Scope for growth however, was not a perfect estimator of actual growth as indicated by the fact that the regression between the two did not intersect the origin. One reason for this is that energy spent for the synthesis of cuticle which was subsequently lost during ecdysis was not accounted for in measurements. However, the error introduced should be small since chitin only makes
up approximately 12% of the cuticle of C. sapidus (Wang and Stickle, submitted). Since energy spent calcifying the cuticle is included in routine metabolism, most of the energy lost has been accounted for in the determination of maintenance expenditure.

Ratios of RNA:DNA and protein:DNA were significantly correlated to both scope for growth averaged over 21 days and actual growth over the same time period. The significant correlations suggest that the ratios do indicate the physiological condition and growth of juvenile blue crabs. The use of RNA:DNA and protein:DNA as indicators of physiological condition and growth has a firm biochemical basis. Increased scope for growth leads to increased growth, as demonstrated in this study. Growth takes place primarily via protein synthesis which requires RNA. The amount of RNA present in a cell is a reflection of the rate of protein synthesis and therefore growth. Since DNA content is usually constant per cell, RNA and protein content is normalized as RNA:DNA and protein:DNA ratios to eliminate differences in cell number or size in tissue samples (Hotchkiss, 1955; Haines, 1973).

Many investigators have suggested the use of changes in nucleic acid content and ratio as indicators of a variety of biological functions. For example, Mudge et al. (1977) followed changes in the pattern of RNA synthesis to infer changes in steroidogenesis in acid stressed brook trout. Sutcliffe (1965, 1970), Bulow (1970), Bulow et al. (1978), Haines (1973) and Dortch et al. (1983) found RNA:DNA ratios to be clearly related to growth rates in a variety of marine organisms. Buckley (1980, 1981, 1984) and Wang and Stickle (1986b) have found the RNA:DNA ratio to be a reliable and sensitive index of nutritional state.
in larval fish and juvenile blue crabs.

The correlation between the RNA:DNA ratio and protein:DNA ratio was highly significant. This suggests that either ratios of RNA:DNA or protein:DNA may be used as indicators of growth rate. This has been true for phytoplankton as demonstrated by Dortch et al. (1984). One advantage of protein:DNA ratios over RNA:DNA ratios is that protein is much easier to measure than RNA.

The scatter in the data and relative low coefficients of determination in the regression of RNA:DNA and protein:DNA ratios against both scope for growth and actual growth should not be overlooked. This is because there are many factors which influence the relationships between nucleic acid content, protein synthesis and growth. It is clear that the usefulness of nucleic acid contents and ratio as indicators of biological processes is limited by the nature of the investigation. Its utility is limited in studying natural populations when the environmental variability is not known since the effects of a host of both environmental and biological factors on nucleic acid synthesis is still unclear (Dagg and Littlepage, 1972; Ota and Landry, 1984). However, if the environmental conditions are known and the analysis is limited to samples of given size and age structure, useful predictions on the physiological condition of the organism may be estimated. For example, Buckley's (1984) model of larval fish growth using larval RNA:DNA ratios is able to account for 92% of the variability when the water temperature is included also.

In conclusion, we found significant dose dependent decreases in growth of C. sapidus exposed to the water-soluble fraction of crude oil. The decrease in growth was due primarily to decreases in energy absorbed. Energy expenditure of crabs exposed to petroleum
hydrocarbons was positively related to the exposure concentration. Scope for growth was inversely related to aromatic hydrocarbon concentration. RNA:DNA and protein:DNA ratios of crabs exposed to the water-soluble fraction of crude oil decreased when compared to those of control crabs. The highly significant correlation between the ratios and measured growth and scope for growth suggests that they may be of use in assaying the physiological condition of experimental organisms.
CHAPTER THREE:  
BIOCHEMICAL COMPOSITION OF THE BLUE CRAB, CALLINECTES SAPIIDUS, 
EXPOSED TO THE WATER-SOLUBLE FRACTION OF CRUDE OIL.

INTRODUCTION

Physiology of organisms are altered when exposed to sublethal levels of pollutant stress. From an energetic point of view, such alterations affect energy intake (e.g. Widdows et al., 1982; Stickle, et al., 1984, 1985; O'Clair and Rice, 1985; Wang and Stickle, 1986a) and/or energy expenditure (e.g. Laughlin and Neff, 1980; Smith and Hargreaves, 1984; Carr and Lindin, 1984; Wang and Stickle, 1986a). The net result of such alterations is a change in scope for growth or energy available for growth and reproduction. Alterations in scope for growth disrupt the pattern of growth at the cellular and ultimately the organismal level. Although changes in physiology (Widdows et al., 1982; Stickle et al., 1984, 1985, 1986) and growth (Edwards, 1978; Cantelmo et al., 1981, 1982; Capuzzo and Lancaster, 1982; Wang and Stickle, submitted) have been documented in organisms exposed to sublethal levels of petroleum hydrocarbons, there is little information on how growth of the organism is affected at the biochemical/cellular level. Are tissue content and constituents altered as well? If so, how are the constituents altered relative to each other?

We have studied the effects of the water-soluble fraction of South Louisiana crude oil on the bioenergetics of the blue crab, Callinectes sapidus, (Wang and Stickle, 1986a). The LC50 was 3927 ± 778 ppb aromatic hydrocarbons. We documented alterations in rates of feeding, respiration and excretion in crabs exposed to sublethal concentrations of aromatic hydrocarbons, culminating in reduced scope for growth. As a consequence of reduced scope for growth, decreased growth and delayed
molting were also observed. Subsequently, we summarized the bioenergetic data in terms of ash free dry weight, examined changes in organic tissue growth and tested whether the ratios of RNA:DNA and protein:DNA could be used as indicators of environmental stress (Wang and Stickle, submitted). In the present study, we have determined changes in protein, lipid, RNA, DNA, chitin and ash content of blue crabs exposed to petroleum hydrocarbons. Our objective was to determine how energy available for growth is partitioned among the various biochemical components and thereby how sublethal stress affect blue crabs at the tissue level. We also analyzed the lipid class composition of crabs to determine how energy available for growth is partitioned between structural and storage forms of lipid.

MATERIALS AND METHODS

Juvenile blue crabs were exposed to the water-soluble fraction of South Louisiana crude oil in the laboratory for 21 days. The flow-through system and procedures used to determine and maintain stable hydrocarbon concentrations have been described in detail previously (Wang and Stickle, 1986a). Aromatic hydrocarbon concentrations of 0, 820 ± 45, 1476 ± 52 and 2504 ± 78 ppb were used. Crabs were weighed and their carapace width measured at the end of the exposure period, quickly frozen in liquid nitrogen and then stored at -70°C.

For biochemical analyses, crabs were pulverized to a powder using a mortar and pestle cooled in liquid nitrogen. An aliquot was processed immediately for nucleic acid and protein analyses. An aliquot was also weighed, dried at 70°C for 24 hr and reweighed to determine water content. The remaining pulverized crab tissue was freeze-dried in a Labconco Freeze Drier-5 for ash, total lipid and chitin analyses.
Ash content was determined gravimetrically. Freeze-dried tissue was ashed in pre-ashed and pre-weighed aluminum pans at 450°C for 12 hours. Ash-free dry weight was calculated as the weight difference between freeze-dried and ashed tissue.

Lipids were extracted from freeze-dried tissue for total lipid determination. The extraction procedure of Bligh and Dyer (1959) was used. Total lipid was determined gravimetrically. Extracts were evaporated to dryness under a stream of nitrogen and then further dried at 65°C for four hours before weighing.

Chitin was analyzed according to Raymont et al. (1964). Freeze-dried tissue was boiled in 50% NaOH for two hours to remove the protein leaving chitinous exoskeleton. These were washed with 0.01 N HCl then twice with water and dried at 70°C overnight. The dried material was termed shell material and was ashed after weighing. The loss in weight was taken to be that of chitin.

DNA, RNA and protein levels were determined colorimetrically. Diphenylamine and orcinol were used as the chromogenic substance for DNA and RNA, respectively (Webb and Levy, 1958). Protein concentration was determined using the Coomassie blue dye binding assay as outlined by Peterson (1983). Calf thymus DNA, yeast RNA and bovine serum albumin were used to construct standard curves for DNA, RNA and protein, respectively. The exact procedures used are described in Wang and Stickle (submitted).

Because of the limited number of crabs that could be exposed to crude oil at one time and to insure against alterations in lipid class composition during tissue storage, crabs from which lipids were extracted for lipid class analysis were obtained during a second exposure experiment. The same experimental procedures were followed to
expose crabs to the water-soluble fraction of crude oil. Lipids were extracted immediately at the end of each exposure period, concentrated and sealed in glass ampules under an atmosphere of nitrogen. Exposure concentrations of $605 \pm 35$ and $2011 \pm 75$ were used.

Lipid class composition was analyzed using an Iatroscan TH-10 Mark III. Separation of the lipid classes was accomplished on type S-II Chromarods. The solvent system dichloroethane:chloroform:acetic acid:isopropanol:acetone (92:8:0.1:0.03:0.03) was used to separate the neutral lipids and chloroform:methanol:water (80:35:4) was used to separate the polar lipids. Results from the Iatroscan were digitized using an HP 3390A integrator. Compounds used to generate standard curves for each of the lipid classes included tripalmitin, cholesterol, dipalmitoyl phosphatidyl (P.) choline, dipalmitoyl P. ethanolamine, P. serine, P. inositol and sphingomyelin. Cholesteryl palmitate, 1,2- and 1,3-dipalmitin, monopalmitin and palmitic acid were also scanned to test for the presence of sterol ester, diacylglycerol, monoacylglycerol and free fatty acids, respectively, in blue crabs. These lipid classes, as well as cardiolipin and lysophosphatidylcholine, were not detectable. Palmitic acid propyl ester was used as the internal standard. All compounds were obtained from Sigma Chemical Co.

It should be noted that lipid attributed to phosphatidylethanolamine also included P. serine and P. inositol. All three lipid classes produced broad peaks that made separation impossible. The peak was attributed to P. ethanolamine because it has been found to be the second most abundant phospholipid after P. choline in the crabs Eriocheir sinensis and Carcinus maenas (Chapelle, 1977). P. choline, P. ethanolamine and sphingomyelin make up at least 78% of
the phospholipids in the various tissues analyzed; if P. serine and P.
inositol were present, the quantity was probably small.

All assays were done in duplicate except for the lipid class
analysis which was done in triplicate. Results were standardized to
crabs of equal weight or size according to Neter and Wasserman (1974)
in order to analyze the effects of crude oil exposure independent of
any differences in crab weight or size. The procedure used has been
described in Wang and Stickle (submitted). Statistical modeling was
performed using the General Linear Models procedure (SAS Institute,
Cary, North Carolina). Duncan's multiple range test was used to test
for significant differences between treatment means within each
sampling date. Differences in treatment means were not tested over
time since the measurements taken were not of the same crabs sampled
over time. All significant differences are given at the \( = 0.05 \) level
or better.

RESULTS

Significant changes in the biochemical composition of *C. sapidus*
took place during the 21 day exposure to the water-soluble fraction of
South Louisiana crude oil. Tissue content, expressed as mg ash-free
dry wt per mm carapace width, varied inversely with exposure
concentration on days 14 and 21 (Fig. 14). The difference in tissue
content between control crabs and crabs exposed to crude oil increased
over time. Tissue content of crabs exposed to 2511 ppb was 89, 64 and
42% of that of control crabs on days 7, 14 and 21, respectively. It
should be noted that growth in both weight and size took place in all
crabs sublethally exposed to petroleum hydrocarbons as reported earlier
(Wang and Stickle, 1986a, submitted). However, the difference in
growth between control crabs and crabs exposed to 2504 ppb was much
Fig. 14. Tissue content of *Callinectes sapidus* exposed to the water-soluble fraction of crude oil. Tissue content was adjusted for standard crabs of 25.2 mm carapace width, the average size of all crabs used. Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different. (mg-ash free dry tissue \( \cdot \) mm carapace width\(^{-1} \); \( n = 8 \) on day 7 and 7 on days 14 and 21)
TOTAL AROMATIC HYDROCARBONS (ppb)
greater in terms of tissue weight than in terms of size, i.e., control crabs grew by 350% in ash-free tissue but only by 60% in carapace width over 21 days while crabs exposed to 2504 ppb total aromatic hydrocarbons grew by 75% in tissue and by 35% in carapace width. Consequently, tissue content (mg ash free dry wt per mm carapace width) increased over time in control crabs, remained stable in crabs exposed to 820 and 1476 ppb aromatic hydrocarbons but decreased in crabs exposed to 2504 ppb.

Significant differences in tissue water content were observed by day 21 (Fig. 15); percent tissue water varied directly with hydrocarbon exposure concentration. Ash-free tissue content was negatively correlated with water content (mg ash-free dry wt · mm carapace width$^{-1}$ = −0.43 (% water) + 40.59, $r^2 = 0.71$, p = 0.001)

Crab tissue was analyzed for protein, lipid, DNA, RNA, chitin, shell and ash in order to determine the relative contribution of each to the change in tissue content as a result of exposure to aromatic hydrocarbons. A significant decrease in ash and RNA content was observed for crabs exposed to 1476 and 2504 ppb by day 7 (Table 4). A significant change in lipid content also took place, but the change did not vary with hydrocarbon concentration. By day 14, a dose-dependent decrease in protein, lipid, RNA, chitin and shell content was observed. By day 21, protein, lipid and RNA content of crabs exposed to crude oil were significantly less than that of control crabs. Protein and lipid content varied inversely with exposure concentration. A significant decrease in chitin, shell and ash was found only in crabs exposed to 2504 ppb aromatic hydrocarbons. DNA content did not vary significantly as a function of exposure concentration over the 21 day period.

Consistent differences in crab tissue components over time were
Fig. 15. Percent tissue water of *Callinectes sapidus* exposed to the water-soluble fraction of crude oil. Percent water was adjusted for standard crabs of 1341 mg fresh wt, the average wt of all crabs used. Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different. (n = 8 on day 7 and 7 on days 14 and 21)
TOTAL AROMATIC HYDROCARBONS (ppb)
Table 4. Biochemical content of *Callinectes sapidus* exposed to aromatic hydrocarbons. Contents were adjusted for standard crabs of 25.3 mm carapace width. (μg·mm carapace width⁻¹ ± SEM; n = 8 on day 7 and 8 on days 14 and 21) Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different.
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<th>Day 7</th>
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<th>Day 21</th>
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<td>6210 ± 771&lt;sup&gt;A&lt;/sup&gt;</td>
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not found when they were calculated in terms of concentration, suggesting that relative constancy in biochemical makeup was maintained even though large changes in tissue content took place (Table 5). RNA concentration of crabs exposed to crude oil was significantly lower than that of control crabs only on days 7 and 14. A decrease in protein and DNA concentration was observed only on day 14. Lipid concentration was significantly lower for crabs exposed to crude oil on days 14 and 21. In general, chitin, shell and ash concentration was higher in crabs exposed to crude oil than control crabs, although the differences were not always statistically significant.

Concentrations of five lipid classes were analyzed in order to determine how each class contributed to the change in total lipid over exposure concentration and time. Significant changes in concentration were found in all lipid classes except for sphingomyelin which remained remarkably stable (Table 6). Concentrations of phosphatidyl choline and phosphatidyl ethanolamine were consistently lower in crabs exposed to crude oil relative to control crabs. Changes in sterol concentration were not consistent. Sterol concentration in crabs exposed to 620 ppb was significantly lower than that of control crabs on days 7 and 14. Sterol concentration of crabs exposed to 2011 ppb was significantly lower than that of control crabs only on day 21. The largest change in lipid concentration took place for triacylglycerol. Although a significant decrease was not observed until day 14, triacylglycerol concentration of crabs exposed to 2011 ppb was negligible by day 21 and its concentration in crabs exposed to 620 ppb was approximately 52% of that of control crabs.
Table 5. Biochemical concentration of *Callinectes sapidus* exposed to aromatic hydrocarbon concentration. Shell and ash concentrations were adjusted for standard crabs of 399 mg dry wt, others were adjusted for standard crabs of 255 mg ash free dry wt. Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different. (ug·mg⁻¹ ± SEM; n = 8 on day 7 and 8 on days 14 and 21)
### Aromatic Hydrocarbon Concentration (ppb)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>820</th>
<th>1476</th>
<th>2504</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>$366 \pm 22^A$</td>
<td>$370 \pm 24^A$</td>
<td>$377 \pm 13^A$</td>
<td>$381 \pm 15^A$</td>
</tr>
<tr>
<td>Lipid</td>
<td>$70 \pm 4^AB$</td>
<td>$70 \pm 4^AB$</td>
<td>$61 \pm 4^B$</td>
<td>$75 \pm 6^A$</td>
</tr>
<tr>
<td>DNA</td>
<td>$2.2 \pm 0.2^A$</td>
<td>$2.3 \pm 0.2^A$</td>
<td>$2.2 \pm 0.3^A$</td>
<td>$2.4 \pm 0.2^A$</td>
</tr>
<tr>
<td>RNA</td>
<td>$11.4 \pm 0.5^A$</td>
<td>$9.7 \pm 1.0^AB$</td>
<td>$9.1 \pm 0.8^AB$</td>
<td>$7.9 \pm 0.5^B$</td>
</tr>
<tr>
<td>Chitin</td>
<td>$53 \pm 4^B$</td>
<td>$54 \pm 4^B$</td>
<td>$70 \pm 8^A$</td>
<td>$61 \pm 4^AB$</td>
</tr>
<tr>
<td>Shell</td>
<td>$299 \pm 16^A$</td>
<td>$324 \pm 23^A$</td>
<td>$341 \pm 26^A$</td>
<td>$310 \pm 8^A$</td>
</tr>
<tr>
<td>Ash</td>
<td>$378 \pm 15^A$</td>
<td>$344 \pm 7^AB$</td>
<td>$333 \pm 16^B$</td>
<td>$375 \pm 10^A$</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>$468 \pm 51^A$</td>
<td>$382 \pm 15^B$</td>
<td>$341 \pm 8^B$</td>
<td>$356 \pm 9^B$</td>
</tr>
<tr>
<td>Lipid</td>
<td>$94 \pm 4^A$</td>
<td>$66 \pm 3^B$</td>
<td>$62 \pm 3^B$</td>
<td>$70 \pm 2^B$</td>
</tr>
<tr>
<td>DNA</td>
<td>$2.5 \pm 0.1^A$</td>
<td>$1.8 \pm 0.2^B$</td>
<td>$1.8 \pm 0.1^B$</td>
<td>$2.1 \pm 0.1^AB$</td>
</tr>
<tr>
<td>RNA</td>
<td>$12.3 \pm 1.4^A$</td>
<td>$9.0 \pm 0.5^B$</td>
<td>$8.4 \pm 0.3^B$</td>
<td>$9.3 \pm 0.4^B$</td>
</tr>
<tr>
<td>Chitin</td>
<td>$38 \pm 7^B$</td>
<td>$65 \pm 8^A$</td>
<td>$69 \pm 4^A$</td>
<td>$66 \pm 5^A$</td>
</tr>
<tr>
<td>Shell</td>
<td>$235 \pm 17^B$</td>
<td>$315 \pm 24^A$</td>
<td>$340 \pm 15^A$</td>
<td>$336 \pm 14^A$</td>
</tr>
<tr>
<td>Ash</td>
<td>$315 \pm 4^B$</td>
<td>$328 \pm 11^B$</td>
<td>$330 \pm 9^B$</td>
<td>$406 \pm 9^A$</td>
</tr>
<tr>
<td><strong>Day 21</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>$400 \pm 37^A$</td>
<td>$432 \pm 19^A$</td>
<td>$370 \pm 34^A$</td>
<td>$419 \pm 17^A$</td>
</tr>
<tr>
<td>Lipid</td>
<td>$81 \pm 8^A$</td>
<td>$68 \pm 3^AB$</td>
<td>$63 \pm 4^B$</td>
<td>$70 \pm 3^AB$</td>
</tr>
<tr>
<td>DNA</td>
<td>$2.1 \pm 0.4^A$</td>
<td>$2.1 \pm 0.2^A$</td>
<td>$2.7 \pm 0.3^A$</td>
<td>$2.5 \pm 0.3^A$</td>
</tr>
<tr>
<td>RNA</td>
<td>$11.5 \pm 0.9^A$</td>
<td>$7.0 \pm 0.7^B$</td>
<td>$10.0 \pm 1.2^A$</td>
<td>$9.9 \pm 0.6^A$</td>
</tr>
<tr>
<td>Chitin</td>
<td>$47 \pm 9^A$</td>
<td>$57 \pm 4^A$</td>
<td>$59 \pm 3^A$</td>
<td>$57 \pm 5^A$</td>
</tr>
<tr>
<td>Shell</td>
<td>$274 \pm 43^A$</td>
<td>$307 \pm 27^A$</td>
<td>$324 \pm 21^A$</td>
<td>$293 \pm 9^A$</td>
</tr>
<tr>
<td>Ash</td>
<td>$327 \pm 32^A$</td>
<td>$365 \pm 21^A$</td>
<td>$324 \pm 11^A$</td>
<td>$387 \pm 10^A$</td>
</tr>
</tbody>
</table>
Table 6. Lipid class composition of *Callinectes sapidus* exposed to petroleum aromatic hydrocarbons. Lipid concentrations were adjusted for standard crabs of 289 mg dry wt. Letters represent Duncan's multiple range test for differences between doses within day; means with the same letter are not significantly different. (ug•mg⁻¹ ± SEM; n = 5) TRI, triacylglycerol; STER, sterols; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine.
<table>
<thead>
<tr>
<th>Day</th>
<th>TRI</th>
<th>STER</th>
<th>PC</th>
<th>SM</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.17 ± 0.19&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.26 ± 0.13&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.94 ± 0.24&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.23 ± 0.06&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.35 ± 0.21&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>1.68 ± 0.11&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.02 ± 0.21&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.79 ± 0.19&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.17 ± 0.08&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.16 ± 0.28&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>620</td>
<td>1.87 ± 0.37&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.62 ± 0.20&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.99 ± 0.45&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.37 ± 0.08&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.69 ± 0.37&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>2011</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>1.94 ± 0.12&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.32 ± 0.14&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4.73 ± 0.17&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.15 ± 0.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.21 ± 0.17&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.09 ± 0.20&lt;sup&gt;B&lt;/sup&gt;</td>
<td>2.92 ± 0.11&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.59 ± 0.20&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.09 ± 0.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.53 ± 0.19&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.61 ± 0.16&lt;sup&gt;C&lt;/sup&gt;</td>
<td>3.32 ± 0.12&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.57 ± 0.14&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.09 ± 0.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.58 ± 0.13&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 21</td>
<td>1.28 ± 0.10&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.49 ± 0.12&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.71 ± 0.28&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.17 ± 0.06&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.55 ± 0.25&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.67 ± 0.15&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.20 ± 0.18&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.92 ± 0.18&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.12 ± 0.07&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.48 ± 0.24&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.07 ± 0.04&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.66 ± 0.17&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3.27 ± 0.17&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.04 ± 0.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.65 ± 0.18&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
DISCUSSION

Crabs exposed to the water-soluble fraction of crude oil had significantly reduced ash free tissue content. Since protein made up 73% of the organic tissue measured, reduction in protein content accounted for the major portion of the difference in tissue content between control crabs and crabs exposed to crude oil. Significant decreases in protein content took place by day 14. By day 21, the protein content of crabs exposed to 820, 1476 and 2504 ppb aromatic hydrocarbons was 71, 63 and 44 % of that of control crabs. Since RNA is produced during gene transcription and is necessary for protein synthesis during growth, the biochemical basis for the reduced protein content was probably the reduced RNA content among crabs exposed to crude oil. RNA content of crabs exposed to crude oil was significantly reduced by day 7 and the reduction in RNA was maintained throughout the 21 day experiment. The positive correlation between RNA and protein content of all crabs was highly significant (protein content = 32.71 RNA content + 43.27; r^2 = 0.76; p = 0.001).

Reduced RNA content has been found to be a consistent response among chronically stressed animals. Examples include fish larvae (Buckley, 1980, 1981) and juvenile blue crabs (Wang and Stickle, 1986b) during starvation, fish larvae exposed to various toxicants (Barron and Adelman, 1984) and brook trout exposed to acid water (pH 4.0) (Mudge, et al., 1977). As a result of the consistent reduction in RNA level during stress, its use as an indicator of stress has been proposed and tested (Wang and Stickle, submitted). Often, DNA content is used to normalize the RNA content, making the ratio of RNA to DNA independent of cell size or mass. We found reduced ratios of RNA to DNA and protein to DNA in blue crabs exposed to petroleum hydrocarbons (Wang
and Stickle, submitted). In addition, RNA:DNA and protein:DNA ratios were significantly correlated to both scope for growth and measured growth, indicating that the ratios may be useful in assaying the instantaneous physiological condition of experimental organisms.

DNA content of crabs exposed to crude oil was not significantly different from that of control crabs and did not vary as a function of exposure concentration. If we assume that DNA content is constant per cell, we must conclude that growth in terms of cell number was not affected. Differences in tissue content then, resulted from differences in cell content.

Chitin and ash content were significantly lower in crabs exposed to 2504 ppb by day 21. Since chitin and ash (inorganic minerals) are principal components of the cuticle, the data indicate that the cuticle of crabs exposed to the highest exposure concentration was significantly less fortified. This is supported by data on shell content which show that crabs exposed to 2504 ppb contained significantly less skeletal material per unit carapace width. There is little information in the literature with which to compare our data. Studies on the biochemical composition of crustaceans have found increased ash concentration as a result of starvation (Ikeda, 1971; Mayzaud, 1976; Anger and Nair, 1979). Such increases were due to the catabolism of metabolic reserves. Our study differs in that all of the crabs grew. The reduction in shell content in crabs exposed to 2504 ppb hydrocarbons resulted from a reduction in the rate of "cuticle growth" relative to growth in size.

There were two shortcomings in this study that should be pointed out. First, carbohydrate levels were not determined. This was because
the quantity of carbohydrate, glycogen in particular, has been found to be minor relative to lipid and protein in crustaceans (Dall, 1965; Schafer, 1968; Armitage et al., 1972). The value of glycogen as an energy reserve in crustaceans is believed to be negligible (Neiland and Scheer, 1953; Schafer, 1968). Second, the effect of the molt cycle was not measured. There was little that could be done to manipulate the molt cycle during the experiment. The sampling dates had been predetermined during the design of the experiment. Fortunately, we did not find significant differences in either the molt stages of the crabs sampled (p > 0.43) or the number of days since molting when the crabs were sampled (p > 0.14) as a function of petroleum hydrocarbon concentrations.

A significant difference in lipid content as a function of hydrocarbon exposure took place by day 14. By day 21, the lipid content of crabs exposed to 820, 1476 and 2504 ppb aromatic hydrocarbons were 61, 58 and 34 % of that of control crabs, respectively. Significant differences in lipid concentration were also observed between control crabs and crabs exposed to crude oil. Our results indicate that crabs exposed to aromatic hydrocarbons not only contain less lipid per unit size but also less lipid per unit ash free tissue. The differences were not due to differential substrate catabolism as a function of aromatic hydrocarbon exposure. We did not find significant differences in the O:N ratio of *C. sapidus* as a function of hydrocarbon exposure (Wang and Stickle, submitted), indicating that protein as well as non-protein metabolic substrates were utilized. We suggest that such differences in lipid concentration reflect differences in energy accumulation and storage. Accumulation and storage of energy in the form of lipids has been documented in a
wide variety of crustaceans (see reviews by Giese, 1966; Morris and Culkin, 1977). We have found scope for growth or energy available for growth and reproduction of crabs exposed to crude oil to be inversely related to the exposure concentration (Wang and Stickle, 1986a). The reduced level of energy available in crabs exposed to crude oil was probably disproportionately channeled into shell and tissue growth. Absorbed energy was probably not channeled toward synthesis of compounds known to be energy reserves, such as triacylglycerol. Similar alterations in the pattern of lipid storage and utilization have been documented by Capuzzo et al. (1984) for larvae of the American lobster, Homarus americanus. Additional research of this nature is warranted. Reduced energy intake has been shown to be a common consequence of sublethal stress in a variety of marine organisms (Johns and Pechenik, 1980; Widdows et al., 1982; Stickle et al., 1984, 1985, 1986). Additional information is needed to delineate the pattern by which energy is partitioned into growth, lipid reserve and reproduction in mature organisms under such conditions.

Significant differences in concentration of all lipid classes analyzed except sphingomyelin were observed between crabs exposed to crude oil and control crabs. Among the neutral lipids, a significant decrease in triacylglycerol concentration took place by day 14. By day 21, triacylglycerol concentration of crabs exposed to 620 and 2011 ppb were 52 and 5% of those of control crabs, respectively. The role of triacylglycerol as a reservoir of potential chemical energy in vertebrates is well established (White et al., 1978) and there is no evidence to suggest otherwise in the invertebrates. Our results parallel those of Capuzzo et al. (1984) who also reported decreased
triacylglycerol levels in larval lobsters exposed to South Louisiana crude oil. It is clear that the decrease in triacylglycerol concentration in crabs exposed to crude oil is due to alterations in the pattern of energy utilization. Available energy is utilized for tissue growth rather than intracellular storage in the form of triacylglycerol reserves.

Sterol concentration of crabs exposed to 2011 ppb hydrocarbons were elevated on day 7 but were significantly less than that of control crabs by day 21. Capuzzo et al. (1984) reported increased accumulation of sterols among larval lobsters exposed to crude oil for 96 hours and suggested that decreased mobilization and/or utilization of cholesterol as a possible reason for the accumulation. This may have been a temporary effect in the present study also. Over 21 days however, decreased sterol concentration may reflect decreased sterol accumulation. The primary role of sterols in biological systems is believed to be as structural components of membranes (Zandee and Kruitwagen, 1975; White et al., 1978). In crustaceans however, storage of sterols in gonada, digestive gland and hypodermis have also been documented (Renaud, 1949; Guary and Kanazawa, 1973). It is believed that the stored sterols are used as precursors of molting and reproductive hormones. If the reduced sterol concentration seen in this study was indeed due to decreased sterol storage, it is possible that this is one mechanism underlying the delay in molting reported earlier (Wang and Stickle, 1986a). Delayed molting in crustaceans as a result of deficiency in sterols needed for B-ecdysone synthesis has been suggested by Anger and Dawirs (1981) and Capuzzo and Lancaster (1982).

Phosphatidylcholine concentration was significantly lower in crabs
exposed to crude oil than control crabs on all sampling dates. The concentration of phosphatidylethanolamine also was significantly lower among crabs exposed to crude oil on days 14 and 21. The ratio of phospholipids (other than sphingomyelin) to sterols among crabs exposed to crude oil was quite consistent ranging from 1.85 to 2.30 and did not show exposure dependent changes. The consistency in the ratio is expected in view of the important role these structural lipids play in regulating membrane fluidity (Shinitzky, 1984). The decrease in concentration of these structural lipids among crabs exposed to 2011 ppb hydrocarbons may reflect the decrease in tissue content of crabs exposed to high crude oil levels. The difference in phospholipid concentration between control crabs and oil-treated crabs is also due to an increase in phospholipid concentration among the control crabs. The reason for this increase is unclear. Sasaki (1984) has found evidence indicating a secondary role for phosphatidylcholine as an energy reserve in larval lobsters. In addition, phospholipids, predominantly phosphatidylcholine (Chapelle, 1977; Chapelle et al., 1979), have now been demonstrated to be the principle circulating lipid among crustaceans (Gilbert and O'Conner, 1970; Lee and Puppione, 1978). The increased phosphatidylcholine concentration among control crabs may indicate phosphatidylcholine storage, perhaps in the hemolymph.

Sphingomyelin concentration of control crabs and crabs exposed to crude oil was not significantly different. Sphingomyelins are structural components of cell membranes but are most abundant in nervous tissue membranes (Houslay and Stanley, 1982). We would expect nervous tissue to be one of the most conserved tissues during crude oil exposure and thus the lack of a difference in sphingomyelin
concentration was not surprising.

In conclusion, we found that although growth took place in juvenile blue crabs sublethally exposed to crude oil, major alterations in tissue composition took place. Tissue content was lower than that of control crabs and was inversely related to exposure concentration. This was due to growth in carapace size despite little increase in tissue content. RNA content of crabs exposed to crude oil was significantly lower than that of control crabs on all sampling dates. Decreased protein content was observed on days 14 and 21. DNA content of crabs exposed to crude oil was not significantly different from that of control crabs, suggesting that the difference in tissue content was due to differences in cell content and not cell number. Decreased chitin, ash and shell content took place among crabs exposed to 2504 ppb, the highest sublethal exposure concentration, indicating a reduction in shell thickness. Significant differences in lipid content and concentration were observed. Analysis of lipid class composition indicates that structural lipids were less affected than were storage forms of lipid among crabs exposed to crude oil.
CHAPTER FOUR:
OVERVIEW, SPECULATIONS AND FUTURE RESEARCH

The primary objective of my dissertation research was to determine how juvenile blue crabs respond to sublethal stress at the organismal level. Tolerance limits of crabs to petroleum aromatic hydrocarbons were determined to establish sublethal exposure concentrations used during subsequent sublethal experiments. *Callinectes sapidus* were extremely tolerant to aromatic hydrocarbons exposure when compared to other species whose long-term tolerance limits are known. The reason for their tolerance is not known. Although the ability of blue crabs to detoxify petroleum hydrocarbons via the cytochrome P-450 system have been reported, the biological significance of this mechanism is still unclear.

Correlation between scope for growth and measured growth over 21 days was highly significant indicating a bioenergetic basis for the reduction in growth among crabs exposed to crude oil. I found a significant inverse relationship between scope for growth and petroleum hydrocarbon exposure concentration. The decrease in scope for growth was due primarily to decreased energy absorption. Since crab food absorption efficiency was not altered as a function of crude oil exposure, the decrease in energy absorption was due entirely to decreased food intake. A number of investigators have reported decreased ability to detect food cues, impaired mobility and decreased feeding motivation in crustaceans exposed to petroleum hydrocarbons. A combination of these factors was probably responsible for the decrease
in feeding among crabs exposed to crude oil in this study.

Although the rate of energy expenditure of crabs exposed to crude oil was not significantly different from that of control crabs, it was significant different between crabs exposed to different concentrations of crude oil. The positive relationship between energy expenditure and exposure concentration may have a behavioral basis. Blue crabs, which are highly mobile, may increase their activity in an effort to escape when exposed to high hydrocarbon concentration but may become quiescent during low level exposure to wait for improved environmental conditions. Additional research which contrasts behavioral responses of mobile versus immobile species to pollutant stress would provide insight in this area.

One of the most perplexing questions I found was the difference in growth in terms of ash-free dry weight versus carapace width as a function of petroleum hydrocarbon exposure. There was a seven fold difference in weight gained between control crabs and those exposed to 2504 ppb over 21 days but only a 66% difference in terms of carapace width. The reason for this is not clear. One possibility is that size increase is an anti-predation adaptation. Blue crabs are highly aggressive. I have noticed that smaller blue crabs held in the laboratory in communal tanks were always the first to "disappear". Selective pressures may have shaped a pattern of growth in which size increase is always maximized during each molt cycle despite little increase in tissue content. Additional research on the pattern of crustacean growth is encouraged, especially factors which control size increase versus tissue growth.
Analyses of the biochemical composition of blue crabs indicate that the decrease in tissue content among crabs exposed to crude oil is due to decreases in cell content and not cell number. RNA content of crabs were sensitive to crude oil exposure while DNA content did not vary. Significant correlations between the ratios of RNA:DNA and protein:DNA and scope for growth and measured growth suggest that these ratios may be useful as indicators of the physiological condition of experimental organisms. Analysis of five lipid classes indicate that structural lipids among crabs exposed to petroleum hydrocarbons were less affected than were lipids known to be important in energy storage. The observed changes in biochemical composition suggest that the pattern of energy utilization was altered in crabs exposed to crude oil. Growth in size took place despite little increase in tissue content and available energy was used for growth with little being stored in lipid reserves.
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25 August 1986

Bob King
Contracts and Rights Manager
University of South Carolina Press
Columbia, SC 29208

Dear Mr. King:

We are writing to obtain copyright release to include a chapter entitled "Bioenergetics, Growth and Molting of the Blue Crab, Callinectes sapidus, Exposed to the Water-soluble Fraction of South Louisiana Crude Oil" in the senior author's Ph.D. dissertation. The chapter will be published by the University of South Carolina Press under the tentative title Pollution and Physiology of Marine Organisms. The book has been commissioned by the Belle W. Baruch Institute for Marine Biology and Coastal Research, of the University of South Carolina.

For your convenience, a copy of this letter may serve as a release-form; the duplicate copy is for your files. Thank you for your assistance.

Sincerely,

Shiao Xu Wang
Ph.D. Candidate

William B. Stickle
Professor

We grant permission for the use requested above.

Robert T. King
Publisher

8/29/86
VITA


Upon the completion of his studies at William Carey College in 1976, he moved across town and entered the master’s degree program at the University of Southern Mississippi. During the Fall of 1977, the author moved to Ocean Springs, Mississippi in order to conduct his thesis research at the Gulf Coast Research Laboratory in the area of physiology of marine organisms. At GCRL, the author held a position as a graduate student research assistant in the Physiology Section which was involved in environmental toxicology at the time. During this period, Shiao Wang completed his thesis on the effects of temperature and declining oxygen tension on the metabolic rate of juvenile brown shrimp, Penaeus aztecus.

Shiao Wang entered the Ph.D. program in the Department of Zoology and Physiology at LSU in August 1981. He was awarded a research fellowship from the LSU Mining and Mineral Resources Research Institute in 1982 and was supported by this fellowship until the end of his tenure at LSU.

The author has accepted a postdoctoral position in the laboratory of Dr. Dorothy M. Skinner at the Oak Ridge National Laboratory, Tennessee.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Shiao Yu Wang

Major Field: Physiology

Title of Dissertation: Tolerance, Bioenergetics and Biochemical Composition of the Blue Crab, Callinectes sapidus Rathbun, Exposed to the Water-soluble Fraction of South Louisiana Crude Oil

Approved:

[Signatures and names]

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures and names]

Date of Examination:

August 29, 1986