Comparison of an Electron Transport System (ETS) enzyme-mediated reduction assay and respiration rate of the invasive copepod Eurytemora carolleae in Green Bay, WI, USA

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Comparison of an Electron Transport System (ETS) enzyme-mediated reduction assay and respiration rate of the invasive copepod *Eurytemora carolleae* in Green Bay, WI, USA

By Alexander W. Timpe

May 2019

A Thesis Submitted in Candidacy for Honors at Graduation
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>i</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Honor Code Reaffirmation</td>
<td>v</td>
</tr>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>The Value of Aquatic Systems</td>
<td>2</td>
</tr>
<tr>
<td>Environmental Stressors: Eutrophication and Climate Change</td>
<td>2</td>
</tr>
<tr>
<td>Environmental Stressors: Aquatic Invasive Species</td>
<td>10</td>
</tr>
<tr>
<td>Lake Michigan and the Green Bay System</td>
<td>15</td>
</tr>
<tr>
<td>Study Organism: <em>Eurytemora carolleae</em></td>
<td>18</td>
</tr>
<tr>
<td>Study Goals</td>
<td>20</td>
</tr>
<tr>
<td>Introduction to Methods</td>
<td>21</td>
</tr>
<tr>
<td>Methods</td>
<td>23</td>
</tr>
<tr>
<td>Collection and Maintenance of Organisms</td>
<td>23</td>
</tr>
<tr>
<td>Measurement of Respiration</td>
<td>24</td>
</tr>
<tr>
<td>Determination of ETS Activity</td>
<td>25</td>
</tr>
<tr>
<td>Determination of ETS : Respiration Ratios</td>
<td>27</td>
</tr>
<tr>
<td>Method Proofing: Incubation Time and Homogenate Dilution</td>
<td>27</td>
</tr>
<tr>
<td>Determination of the Arrhenius Activation Energy (<em>E_A</em>)</td>
<td>28</td>
</tr>
</tbody>
</table>
Results

Respiration Rate

ETS Assay Method Proofing

Effect of the Respiration Experiment on ETS Activity

Incubation Time and Homogenate Dilution

ETS Activity of *E. carolleeae*

ETS : Respiration Ratio

Arrhenius Activation Energy (\(E_A\))

Discussion

Points of Emphasis

Interpretation of Results in a Broader Context

Respiration Rate

ETS Activity and the ETS : Respiration Ratio

Arrhenius Activation Energy (\(E_A\))

Conclusions and Implications

References Cited
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 1</td>
<td>Respiration Rate of <em>E. carolleeae</em> and <em>S. oregonensis</em></td>
<td>29</td>
</tr>
<tr>
<td>Fig. 2</td>
<td>Effect of the Respiration Experiments on ETS Activity</td>
<td>30</td>
</tr>
<tr>
<td>Fig. 3</td>
<td>Corrected Optical Density (COD) Over Incubation Time</td>
<td>31</td>
</tr>
<tr>
<td>Fig. 4</td>
<td>Calculated ETS Over Incubation Time</td>
<td>32</td>
</tr>
<tr>
<td>Fig. 5</td>
<td>Calculated ETS Activity for <em>E. carolleeae</em></td>
<td>32</td>
</tr>
<tr>
<td>Fig. 6</td>
<td>ETS : Respiration Ratio for <em>E. carolleeae</em></td>
<td>33</td>
</tr>
<tr>
<td>Fig. 7</td>
<td>Arrhenius Plot for <em>E. carolleeae</em> metabolic enzymes</td>
<td>34</td>
</tr>
</tbody>
</table>

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I hereby reaffirm the Lawrence University Honor Code

Alexander W Tingfe
Abstract

The use of aquatic resources for agriculture, trade, and recreation adds stress to water-dwelling organisms. Rapid changes in abiotic conditions, such as warming due to climate change and nutrient loading from agricultural runoff and urban areas, threaten to induce profound alterations to aquatic environments. These changes affect interspecific community interactions and may cause an aquatic resource to lose its functionality that is valuable to humans. Studying organisms such as plankton that form an ecosystem’s foundation is an important step towards understanding the entire food web and predicting how it may or may not be able to respond to a changing environment. One important planktonic species in the Laurentian Great Lakes is the invasive calanoid copepod *Eurytemora carolleae* (formerly considered part of the *Eurytemora affinis* species complex). This study analyzes the metabolic activity of *E. carolleae* in Little Sturgeon Bay, WI, USA using two different methods, over a range of temperatures from 9º to 26ºC. Total oxygen consumption was measured directly using a micropulse oxygen probe, and the activity of aerobic metabolic enzymes in the electron transport system (ETS) was quantified using the *in vitro* reduction of iodonitrotetrazolium chloride (INT). We find that the respiration rate of *E. carolleae* increases linearly from 9º to 26ºC. We also find that the copepod’s metabolic enzymes have an Arrhenius activation energy of 11.1 ± 3.7 kJ/mole and experience a thermal maximum between 22º and 26ºC. This thermal limit has implications for the future success of this species, as the combination of warmer temperatures and the disappearance of oxygenated colder-water refuges may limit *E. carolleae*’s success in the Green Bay system.
Introduction

The Value of Aquatic Systems

The use of aquatic resources for agriculture, trade, and recreation adds stress to water-dwelling organisms. Rapid changes in abiotic conditions, such as warming due to climate change and nutrient loading from agricultural runoff and urban areas, threaten to induce profound alterations to aquatic environments. These changes affect interspecific community interactions and may cause an aquatic resource to lose its functionality that is valuable to humans. Studying organisms such as plankton that form an ecosystem’s foundation is an important step towards understanding the entire food web and predicting how it may or may not be able to respond to a changing environment. One important planktonic species in the Laurentian Great Lakes is the invasive zooplankton *Eurytemora carolleae* (formerly considered part of the *Eurytemora affinis* species complex). My study of this calanoid copepod’s metabolic activity in Little Sturgeon Bay, WI, USA, analyzes the organism’s response to some of these anthropogenic perturbations – particularly the consequences of nutrient loading and climate change – as a model system to illustrate how similar organisms may or may not be able to respond to a rapidly changing environment.

Environmental Stressors: Eutrophication and Climate Change

Eutrophication, or the increased productivity and simplification of biotic communities as a result of nutrient loading, is one of the most common and well-documented effects that humans have on aquatic environments (Wetzel, 2001 and Carlson, 1977). Although lentic systems (still, fresh water such as lakes) undergo a natural process of eutrophication as the community matures, increased rates of change in most areas are the direct result of the addition of byproducts from
human industrial and agricultural practices into aquatic systems. Phosphorous- and nitrogen-rich waste from manufacturing, wastewater treatment, agriculture, and urban areas, act as fertilizer for primary producers such as phytoplankton (free-floating microscopic plants and bacteria) and macrophytes (macroscopic, usually rooted aquatic plants). This fertilization increases the productivity (amount of atmospheric carbon fixed into sugars per area or volume) of the photosynthesizing community, which in turn increases the potential food availability for organisms that occupy higher trophic levels. However, despite the fact that there may be more reduced organic carbon available for heterotroph consumption, eutrophied areas are characterized by a reduction in the diversity and overall complexity of the aquatic communities at every level, starting at phytoplankton and then propagating into higher trophic levels.

The dominant effect of eutrophication on phytoplankton communities is a shift in community structure. All other factors being equal, in low-nutrient, or “oligotrophic” environments, there is a much greater diversity of phytoplankton phyla. Watson et al. (1997) found that when the amount of phosphorous is low, there is a near equal distribution in plankton biomass between six major phylogenetic groups: green algae (*Chlorophyta*), blue-green algae (cyanobacteria), diatoms (*Bacillariophyceae*), golden algae (*Chrysophyceae*), *Dinophyta* (dinoflagellates), and *Cryptophyta* (a polyphyletic group that employ multiple methods of gaining energy, collectively known as cryptophytes). However, when phosphorous concentrations increase (also known as “phosphorous loading”), the community becomes dominated by blue-green cyanobacteria.

This shift can be explained in part by an important theoretical framework in plant ecology known as Liebig’s Law of the Minimum, which postulates that the growth rate of plants and algae is determined by the scarcest resource relative to need. Because the ratio of major nutrients
in algae (also known as the Redfield ratio: somatic carbon:nitrogen:phosphorus (C:N:P) = 106:16:1) does not change appreciably due to environmental conditions, it is the ratio of those important nutrients that matters as well as their absolute abundance. Phosphorous is typically the limiting nutrient in freshwater systems because, unlike C or N, it does not have a volatile or gaseous phase that allow is to be replenished from the atmosphere into terrestrial or non-marine aquatic systems. Therefore, the only appreciable natural source of P is the erosion of bedrock on geological timescales (Schindler et. al., 1974). When anthropogenic phosphorous loading does occur the N:P ratio typically decreases, and the phytoplankton community is released from phosphorous limitation, with nitrogen becoming the limiting nutrient.

To cope with low-nitrogen environments, many species of cyanobacteria have specialized cells called heterocysts that can fix nitrogen from its gaseous form (N≡N) to ammonium (NH₄⁺). Ammonium is known as a “bioavailable compound,” meaning it can be directly metabolized and incorporated into nitrogen-rich macromolecules such as DNA or the amino acids that make up proteins. Since those cyanobacteria can avoid being nitrogen-limited by producing their own, they can out-compete other types of phytoplankton and become the dominant type.

This change in phytoplankton structure is critically important to most heterotrophic planktonic organisms (collectively known as zooplankton). Many common cyanobacteria, including species in the phyla Dolichospermum (formerly classified as Anabaena), Nodularia and Microcystis, can create a variety of toxins that can be incredibly harmful to animals. They also typically display a variety of anti-predator morphologies, such as colonial forms or mucus sheaths, that make them harder to ingest. For an herbivorous zooplankton, this has two major implications. First, the food that is available takes more energy to consume and second, unless the organism has developed an immunity to the cyanotoxins, much if not all of the energy that
may be gained by eating the cyanobacteria is immediately expended in the metabolism of the toxins or the repair of damaged cells. This affects the zooplankton community by selecting for only those species that can avoid consuming the cyanobacteria (which excludes many passive filter feeders) or that are not affected by the cyanobacteria’s defenses. *Eurytemora* is an example of such an organism.

Copepods such as *Eurytemora* are more selective in their prey choice and are able to better manipulate prey that they capture as compared to common filter feeders such as the water flea *Daphnia*. The long filamentous chains of *Dolichospermum* and *Nodularia* physically interfere with the feeding structures of *Daphnia*, and therefore the animal has to expend much more energy mechanically cleaning its feeding combs and rejecting those prey items that it cannot ingest. Copepods, on the other hand, are much more dexterous, selective feeders that can manipulate the strands such that they become much more economical (i.e. less energy intensive) to consume. Additionally, when comparing the response of *Eurytemora* and *Daphnia* to the toxins produced by these cyanobacteria, it has been observed that the copepod is not nearly as affected. Lampert (1985) found that *Daphnia* mortality rate increased when a population was fed the toxic cyanobacteria *Microcystis* as compared to a control group that was starved, indicating that the zooplankton would be “better off” not eating the food source as opposed to poisoning itself while consuming food. When Engström-Öst et al. (2017) studied the response of *Eurytemora* to cyanotoxins in a similar experiment, they found that as long as the copepods could consume the cyanobacteria during exposure to the toxins, they were able to compensate for the energy loss and showed no significant changes in survivorship as compared to a diet of easily-consumed, nontoxic green algae. Together, these two case studies illustrate how the effects of anthropogenic eutrophication resonate throughout the food web, and how the
phytoplankton community changes can affect the entire aquatic community. A change in the wild that reproduced the effects of those two studies would result in a community shift towards copepods and away from *Daphnia*.

Another important result of eutrophication can be “boom-bust” productivity cycles that are typified by a sequence of phytoplankton blooms, nutrient depletion, mass die-offs, and intense microbial decomposition leading to oxygen reduction. Algal blooms occur when a large amount of excess nutrients allow for exponential growth without a proportional increase in predation from herbivores. When this happens, a large amount of photosynthetic biomass accumulates in the water, effectively shrinking the photic zone (the portion of the water column extending downward from the surface with enough light to support photosynthesis). Large mats of phytoplankton at the surface can shade out other photosynthesizing organisms that are suspended in the water column. This process has two main mechanisms that lead to the same outcome: oxygen depletion.

The first factor leading to oxygen depletion during algal blooms is the conversion of algae that would normally be oxygen producers when light is available into oxygen consumers in low-light conditions. Algae have a basal metabolic rate (amount of reduced organic carbon oxidized per time that is used to maintain cellular processes) that is negative, signifying net oxygen production when photosynthesis is happening. However, when those cells are shaded, they become an oxygen sink that can deplete the water column and cause a hypoxic (low-oxygen) or anoxic (no-oxygen) state. The second mechanism occurs during mass die-off stage of a phytoplankton bloom when the large number of cells deplete the water of nutrients and begin to die as a result. Microbial decomposition of the dying algae increases and can contribute to establishing and maintaining a hypoxic or anoxic state, especially near the sediment-water
interface (Klump et. al., 2018). Since zooplankton and nekton (larger animals such as fish) are obligate aerobes (require oxygen for aerobic metabolism), they will suffocate and die under prolonged exposure to hypoxic or anoxic conditions.

Climate change can alter the niche space available for organisms by pushing water temperatures above some species’ thermal tolerances and exacerbate low-oxygen conditions by increasing the severity and duration of stratified conditions in aquatic ecosystems. Stratification of a water body means that there is a physical barrier, usually driven by density differences, that separates the water column into three sections, each with distinct biological, chemical, and biogeochemical characteristics that are heavily influenced by seasonality and trophic status. From the surface to the bottom, these sections are known as the epilimnion (“top water”), metalimnion (“middle water”; transition zone between the epi- and hypolimnion), and hypolimnion (“bottom water”).

For temperate regions in the northern hemisphere (such as in Little Sturgeon Bay), the winter months are characterized by cold temperatures and permanent or semi-permanent ice cover. During this period, the water column is known as “inversely stratified,” where the coldest water is found in the epilimnion directly underneath the ice, with the rest of the water column stable around 4°C (the temperature at which water is the densest in the liquid phase). During the winter months, oxygen and other nutrient concentrations usually remain high, as microbial decomposition, respiration rates of plankton, and poikilotherm growth rates are depressed due to the low temperatures. Additionally, since oxygen solubility increases in water with decreasing temperature, water oxygen concentrations in cold water can be higher than in warm water. At 4°C, Benson and Krause (1980) found that the oxygen saturation point (the point at which the maximum amount of O₂ is dissolved in water) is 13.107 mg/L. During the summer, when the
surface waters can reach 22°C or higher, the saturation point drops by nearly one third to 8.743 
mg/L.

After the ice melts in the spring, the inverse stratification is broken by a process called 
“spring mixis,” during which mechanical energy derived from wind can circulate the entire (or a 
large part of) the water column. As the water mixes, oxygen is replenished at the surface and 
nutrients are circulated at every depth, leading to intense phytoplankton growth (especially green 
algae and diatoms) that is spurred by the warming temperatures and increased light availability. 

Nutrient-rich terrestrial runoff can also contribute to the nutrient pool. This explosion in 
productivity is followed by a corresponding increase in zooplankton, especially large filter 
feeders such as *Daphnia* that can quickly grow and reproduce in the food-rich and predator-poor 
environment. However, in mid- to late spring during a time known as the “spring clearwater 
phase,” algae populations crash due to a combination of nutrient limitation and intense predation 
from zooplankton. A reduction in zooplankton population directly follows this precipitous drop 
in algae abundance as a result of a lack of food and increased predation pressure from 
planktivores (heterotrophs that consume plankton). In Green Bay and Little Sturgeon Bay these 
include larval fish, large predatory zooplankton (such as *Leptodora kindtii* or *Bytotrephes 
longimanus*) and filter-feeding fish (De Stasio, unpublished data).

In the late spring to early summer, stratification is reestablished as solar radiation heats 
the surface water. The summertime characteristics of the stratification are heavily dependent on 
the trophic status of the water body (Wetzel, 2001). In oligotrophic lakes where microbial 
decomposition is not as intense, the hypolimnion remains cold and well-oxygenated from its 
saturation during spring mixis. In contrast, the oxygen in the hypolimnion of eutrophic (lakes 
with high nutrient concentrations and a productive plant community) and hypereutrophic
(extremely eutrophic) lakes becomes depleted in a matter of weeks by intense microbial decomposition of organic matter (Seto et. al, 1982, in Wetzel, 2001). This structure, typified by an oxic epilimnion and an anoxic hypolimnion, is known as “clinograde.” During the summer, nutrient limitation (especially P) and high temperatures drive the community to be mainly dominated by cyanobacteria, which, coupled with high predation pressure from planktivores, causes a shift in the zooplankton community towards smaller-bodied animals that are more successful at escaping predators.

Stratification is broken during “fall mixis” when the surface waters cool enough such that the density difference between the epilimnion and hypolimnion can be overcome by wind energy. This mixing allows recycled nutrients that were trapped in the hypolimnion to be recirculated into the photic zone. The colder temperatures and water replete with nutrients (especially P and Si) allow the community composition to shift away from cyanobacteria and towards dominance by diatoms. One effect of climate change would be the delay of fall mixis, which would extend the period of dominance by cyanobacteria and prolong the low-oxygen state in the hypolimnion of eutrophic water bodies. Because water becomes stratified in the summer, there are disparate effects of eutrophication and climate change depending on the position in the water column that an organism usually inhabits. Sessile benthic (bottom-dwelling organisms such as macrophytes and mollusks), and epibenthic (organisms that live just above the sediment-water interface such as some fish and zooplankton, including *Eurytemora*) species are much more susceptible to habitat change than organisms that inhabit the continuously-oxygenated surface waters. For those organisms that live in the epilimnion, light limitation and nutrient depletion will be less likely. Additionally, shallower water near the shoreline that is perturbed by
wave action will also be less affected, as the moving water will help to circulate oxygen-enriched water towards the sediment.

**Environmental Stressors: Aquatic Invasive Species**

The success of aquatic invasive species is corollary to the increased productivity and community simplification that is brought about by anthropogenic eutrophication of aquatic resources. Those stressors reduce an ecosystem’s resilience against invasion by opening up potential niche space and selecting for generalist species that may be out-competed by a nonnative specialist. The term “invasive species” has two distinct definitions. The biological definition defines an invasive species as one that is expanding outside its native range (usually mediated by humans), establishing populations in new locales, and disrupting that area’s ecosystem. Under this definition, not all invasive species are non-native (i.e. come from a different place), and not all non-native species are invasive. A native species may become invasive in its native ecosystem if its function in that ecosystem (i.e. its niche space) changes. For example, gut bacteria in humans could be considered invasive if they develop pathogenic qualities or toxins that harm other microorganisms or their host. Additionally, a non-native species may not develop invasive qualities if it assumes a function in the ecosystem that was not previously performed, such as using an unused energy source or habitat, or if it never gains a competitive advantage over established native species. The second, political definition of an invasive species is one that is non-native and harms the environment, economy, or human health. This definition stresses the importance of functionality in determining the invasive status of a species.
A good case study to illustrate the difference between the political and the biological definitions is the invasion of zebra mussel (*Dreissena polymorpha*) into Lake Winnebago, USA. In other places in the Great Lakes and their watershed (such as Green Bay), this mollusk has induced drastic changes to the benthic and planktonic community structure by out-competing native mollusks for space and by removing an incredible volume of food from the water column (e.g. De Stasio et. al., 2014). Because the mollusk is a voracious phytoplanktivore and because it selects for green algae, it has heavily biased the phytoplankton community towards diatoms and cyanobacteria, which, as discussed earlier, has implications for higher trophic levels. It also causes millions of dollars per year in damages to human infrastructure (O’Neil, 1997). In such a case, it would be classified as invasive under both the biological and political definitions.

However, in the case of Lake Winnebago, a longitudinal study by De Stasio (unpublished data) found that there was no significant change in water clarity, phytoplankton biomass, or *Daphnia* abundance after its introduction. Due to the lake’s soft, mucky bottom (which is not habitable by *Dreissena* as the species needs a solid holdfast) and hypereutrophic status, the small population that has been established there has not been able to establish a top-down control on the phytoplankton population and therefore may not be considered “invasive” under the biological definition. However, because many of the surfaces colonized are docks, water intake and outlet pipes and boat hulls, they can significantly impair the human economy around Lake Winnebago by fouling human structures. Due to this economic impairment, they would be considered invasive using the political definition. Because the focus of my study is on the species *Eurytemora carolleae* and how it will interact with its biological community in the face of environmental perturbation, I will primarily consider the status of this invasive species using the biological definition, as it prioritizes ecosystem function over human impact.
A useful conceptual framework for thinking about the likelihood of invasion is the “tens rule” (Jarić and Gorčin, 2012). This describes the general trend where for every 100 foreign species that escape into the wild, 10 will become naturalized in their new environment and create self-sustaining populations. Then, out of those 10 naturalized species, one will become invasive. The success rate of invasive species is also modulated by factors in the environment, principally native species diversity and environmental conditions. First, the abiotic conditions such as salinity, temperature, light and nutrient availability, and seasonal changes, need to be compatible with the invading species. For example, if a species is transported from the arctic to the tropics, it may not be able to survive due to a limited thermal tolerance. Second, it is generally easier to invade “simple” ecosystems, in which there is a small number of species that occupy each trophic level (also known as low diversity). When this is the case, such as in many northern-latitude climates, there is a lower chance of competition with native species. Additionally, simple ecosystems are more subject to damage from anthropogenic disturbance, and that lack of resilience makes potential openings in the environment more common. Physiological aspects such as earlier spawning times, more efficient resource use, ability to exploit an uncontested food source, and faster growth and reproduction are all common traits that invasive species have that allow them to have a competitive advantage and exploit openings in the existing trophic structure and niche space. On a more ecological level, the Enemy Release Hypothesis (ERH) (Keane and Crawley, 2002) posits that a species may become invasive when there are no predators in the introduced area to keep the population in check. When a species experiences low or no predation pressure, it can more efficiently grow, forage, and reproduce, all of which give it a competitive advantage over native species that are subjected to predation pressure.
Once established in an area, invasive species are incredibly costly in both time and capital to remove. This is complicated in aquatic systems by the relatively rapid and wide-ranging dispersal of planktonic larvae. A planktonic stage is an important life history feature of many organisms, including those that are normally stationary as adults (such as mollusks). While it may be possible to remove all of the adults from an area, filtering all of the viable eggs and juveniles out of the water is generally not feasible. Additionally, the biological factors that allow a species to become invasive can inhibit efforts to remove it. If the invasive species became established in a new area despite competition or predation from native species, it is unlikely that, once dominant, those native species will be able to drive it from the system.

To combat invasive species, there are several common methods whose success is dependent on the features of the species being targeted. One of the most common ways to remove a problem species is culling, either manually or chemically (Carlton, 2003; Pyšek and Richardson, 2010). This is much more effective when the population is small, or when the species’ range is limited. However, due to physical constraints of working in aquatic systems, direct, specific attacks are often not as effective. For example, only a single example of successful eradication of zebra mussels in an invaded lake has occurred (Wimbush et al., 2009). This was possible because the initial colonization was detected early by SCUBA diving clubs and massive culling efforts by divers were able to stop the invasion. In comparison to terrestrial systems, species must be trapped using “blind” techniques such as fishing or trapping, as compared to visual techniques such as hunting or specific removal of a certain plant, which can result in heavy damage to non-target species. The use of pesticides, toxins, or other chemicals similarly affects non-target species, since dispersion of toxic compounds is much more rapid in water than over land of through soil, and since the range of that dispersal is much more
challenging to control. However, if a chemical is developed that is specific to the invasive species but unharmful to native ones, that increased dispersal by water could help facilitate culling by expediting its diffusion.

Other more “experimental” methods in invasive species mitigation include biological control (biocontrol for short) and altering the abiotic environment (such as removing habitat) to make it incompatible with the target species. Biocontrol is the process by which the natural predator or a natural competitor to a problem species is introduced into an area to control it. One of the most successful implementations of biocontrol is the mitigation of the prickly pear cactus \((\text{Opuntia stricta})\) in Australia by introduction of its natural predator the \(\text{Cactoblastis} \) moth \((\text{Cactoblastis cactorum})\) (Hosking et. al., 1988). In the Great Lakes, predatory fish such as salmon, walleye, bass, and northern pike have been stocked or introduced to control booming populations of invasive fish including rainbow smelt and alewife (USACE, 2012 and references therein). However, there is always the risk that the biocontrol species can itself become invasive by either competing with, displacing, or preying upon native species.

In the North American Great Lakes, the most common source of invasive species is ballast water from transoceanic vessels. In order to maintain their stability when not carrying cargo, large ships such as tankers, cargo ships, or ocean liners intake large quantities of ballast, usually water, from their port of departure. This volume of water is carried thousands of miles and then discharged in a new location when the ship is loaded with new cargo. During this process, a large variety of aquatic hitchhikers such as plankton, larval fish and crustacea, or benthic species such as macrophytes or mollusks are also transported from their native range and injected into foreign, but potentially suitable habitats. In addition to those species carried in the ballast water, biofouling organisms (those organisms that grow on top of human substrate such
as ship hulls or pipes) including mollusks such as *Dreissena* or barnacles can be carried long distances and then re-establish themselves in a foreign body of water. Over the course of a year, a single ship typically takes in and discharges water multiple times in multiple continents (Holeck et. al., 2004). Another important source of invasive species is fishing equipment or lines, which can snag certain types of voracious planktonic predators, such as fishhook and spiny water fleas. Once across the ocean, secondary dispersal of non-native species across the continent occurs via similar vectors, although overland dispersal on boat trailers, fishing equipment, bait buckers, or in bilge water are also concerns. Many of the most important Great Lakes invasive species, including *Eurytemora*, have native ranges in Asia or Europe. The large volume of intercontinental shipping, especially in the wake of the collapse of the Soviet Union in 1991, helps explain the high species exchange rate between those two continents and freshwater ports in the Great Lakes.

*Lake Michigan and the Green Bay System*

Lake Michigan is the second-largest of the Laurentian Great Lakes by volume. Green bay is the largest embayment in Lake Michigan, covering an area of 145 km² and containing 1.4% of the lake’s water. One important geological feature of Green Bay is the unique shape and bathymetry of its southern half, which acts like a trap that collects sediment and organic material (Klump et. al., 2009). Another important part of the Green Bay system is the Fox River, which drains 40,000 km² of productive Wisconsin farmland, ranches, and urban areas. The river discharges its nutrient-rich waters into the southern tip of Green Bay (also known as the “lower bay”), which causes hypereutrophic conditions in the south. This productive river water is the source of approximately one-third of the nutrients that enter Lake Michigan, which is otherwise a
mesotrophic (moderately productive) or oligotrophic system. Mixing of the nutrient-rich Fox River water and the relatively depleted Lake Michigan water at the Bay’s northern extent creates a steep trophic gradient from south to north, which provides a wide range of different habitats for fish and plankton in a relatively small geographic area (De Stasio et al. 2018 and citations within).

Phytoplankton productivity in Green Bay is complicated by strong bottom-up effects from the rapid recycling of phosphorous by the invasive zebra mussel *Dreissena polymorpha*, which promotes the growth of cyanobacteria by decreasing the available N:P ratio. The bay is also moderated by top-down effects by the spiny water flea (*Bythotrephes longimanus*), an invasive predatory zooplankton that has, when abundant, been shown to decrease the biomass of herbivorous zooplankton by over 99% (Merkle and De Stasio, 2018). In sum, the combined effects of those two species have mitigated the expected improvements in water quality due to reductions in nutrient loading by creating a chemical environment that is conducive to cyanobacterial growth and by removing herbivores that would graze down the booming algal population (De Stasio et al., 2008, 2014; Qualls et al., 2013).

The interaction between the sediment-trapping morphology of the Bay and its high production can lead to oxygen-depleted (<5mg/L) or hypoxic conditions (<3mg/L) for large areas of water. The area of Green Bay closest to the mouth of the Fox River is very shallow (<3m in depth), which promotes mixing by wind and hinders thermal stratification. This constant mixing further increases the productivity of this area by constantly resuspending nutrient-rich sediments and keeping the water well-oxygenated. However, further north in deeper parts of the bay where thermal stratification is more common, the microbial decomposition of the large influx of algae-derived organic matter from the hypereutrophic Fox River outflow can cause
oxygen in the hypolimnion to be quickly depleted. Klump et. al. (2018) found that stratification in Green Bay can last continuously for two months in the summer, leading to a 500km² oxygen-depleted zone in the middle of Green Bay. This oxygen-poor zone roughly the size of Chicago has several implications for the bay’s ecology. The first and most important impact is the relatively low diversity of benthic organisms such as fly larvae and native mollusks. Additionally, this water can be mobilized by a process known as an internal seiche, in which wind or changes in atmospheric pressure can cause the water in the bay to oscillate in its basin, like water sloshing back and forth in a bathtub. This physical process can rapidly advance oxygen depleted water into new areas, which can quickly kill many organisms, especially sessile or slow-moving ones that cannot escape the advancing oxygen-poor environment.

The study site, Little Sturgeon Bay, is a small (approximately 3km long and 1km wide), relatively shallow (~4.5m deep) inlet in the middle of Green Bay, just to the north of the most hypoxic areas reported by Klump et. al. (2018). Although the shallow depth of Little Sturgeon Bay makes wind-modulated mixing more likely than in deeper areas of the Green Bay system, Poli (2015) found that stratified conditions can occur intermittently in Little Sturgeon Bay during summer months. These stratified conditions, as discussed earlier, have the potential to create low-oxygen bottom water, especially in and directly above the sediment. Additionally, because of the proximity of the inlet to commonly hypoxic water, seiches may draw cold, relatively anoxic water into the bay or into its hypolimnion. The hypereutrophic conditions, prolonged stratification, and oxygen depletion are projected to become more severe in the future due to increased human nutrient loading and climate change (Klump et. al., 2018). One organism that may be affected by these drastic changes in abiotic conditions is this study’s subject, the invasive copepod *Eurytemora carolleae*. 
Study Organism: Eurytemora carolleae

_Eurytemora carolleae_ (Alekseev & Souissi, 2011) was, until recently, thought to be part of the _Eurytemora affinis_ species complex (Poppe, 1880). A species complex is a group of very closely related organisms that have nearly identical morphologies, which makes the boundaries between different species ambiguous. However, using molecular techniques, behavioral and reproductive success studies (Lee, 2000), and careful microscopy (Alekseev & Souissi, 2011), this distinct North American variety of _Eurytemora_ has been well characterized and exhibits differences from other populations.

Part of what makes this copepod a successful invasive species is its evolutionary history and life history strategy (how an organism grows from an egg to a reproductive adult), making it resilient and adaptable. The ecology and life history of _E. carolleae_ changes seasonally and by location. Populations generally peak once per year in the Great Lakes, but the occurrence of that peak varies from early spring to fall based on the population and the dynamics of the system (Balcer et. al., 1984; Torke 2001). Adults vertically migrate towards the epilimnion at night (Poli, 2015) and are epibenthic during the day (Evans and Stewart, 1977, in Balcer et. al., 1984). Knatz (1978) and Kimmel and Roman (2004) reported similar findings in populations in Chesapeake Bay, USA. In natural assemblages in the Great Lakes, the population disappears in the middle of the summer when water temperatures reach 24°C. Lab experiments have shown that at temperatures above 22°C, growth is maximized but the number and quality of offspring produced drop dramatically (Lloyd et. al., 2013). This suggests that temperatures below 20°C are optimal for _E. carolleae_. Animals mature from eggs to reproductive adults in 11-37 days, dependent on temperature and can produce up to 34 sexual eggs per day (Kipp, 2006). This
species produces diapausing (“resting eggs”) in the fall. These robust eggs can survive digestion by fish and can remain viable in the sediment for 10-18 years, even in anoxic conditions.

Research on the diet of planktivorous fish by Faber and Jermolojev (1966, in Balcer et al., 1984) suggest that *E. carolleae* is an important or possibly even a preferred food source for many species. Qualitative observations during this study suggested that *E. carolleae* were also popular food sources for predatory or omnivorous copepods such as *Macrocylops, sp.*, *Mesocyclops edax*, and *Acanthocyclops vernalis*. Those species were observed directly feeding on live *Eurytemora*, and *Eurytemora* populations decreased much more rapidly than other small calanoid species such as *Skistodiaptomus oregonensis* or cladocerans such as *Ceriodaphnia, spp.* *E. carolleae* were easier to catch using a pipette (which can be used as a proxy for fish predation success), swam more slowly and were considerably less transparent than other species of copepods enumerated above.

In addition to their introduction to the Great Lakes in the 1950s, there have been many recent successful invasions of *Eurytemora* into freshwater areas in Europe, Japan, the Mississippi River System, and landlocked reservoirs in the United States (Lee, 1999 and Kipp, 2006). *Eurytemora* is what is known as a “euryhaline” species, meaning that it can inhabit a wide variety of salinities (Lee et al., 2003). In its native environment in brackish coastal estuaries and salt marshes (Lee 1999), salinity may change rapidly over the course of the day according to tides, or seasonally with changing fluxes of freshwater from the land. Lee and Peterson (2003) found that individuals were able to acclimate to gradual increases in salinity but were unable to cope with a freshwater environment unless they were genetically predisposed to do so. The consequence of this finding is that invading populations of *Eurytemora* represent a small genetic subset of the greater population that can become isolated in their new habitat and may be
pressured to diverge into a new species or become reproductively isolated. This hypothesis was supported by previous genetic work by Lee (1999) that indicated that invading populations were genetically distinct from each other, further suggesting that it is the wide adaptability intrinsic to *Eurytemora* that makes it a successful invader instead of the happenstance adaptation of one isolated population.

*Study Goals*

This study’s primary concern is the respiration rate of *Eurytemora*. Respiration rate is one of the most important ecological measurements, as it is a proxy for the organism’s energy needs (i.e. how much food is required to maintain body mass, growth, and reproduction). Because metabolic rates in poikilotherms (“cold-blooded” creatures including plankton, algae, and fish) are heavily dependent on temperature, changes in seasonal patterns of warming and cooling by perturbations such as anthropogenic climate change may result in direct impacts on every level of aquatic communities. These perturbations can change the competitive balance of some creatures for a shared resource or cause a physical barrier (such as water temperatures above a creature’s thermal tolerance) that prevents a species from inhabiting a certain area. By measuring *Eurytemora*’s metabolic activity at a variety of temperatures and comparing it to a competitor species, we can predict how its potential range and population size may change in a warming future. A long-term goal of this work is assessing how other human-caused abiotic changes such as eutrophication may work in conjunction with metabolic thermal responses in *Eurytemora* to alter its functionality in the Little Sturgeon Bay system.
Introduction to Methods

Respiration rate is one of the most important ecological measurements, since it is a direct measurement of an organism’s metabolic activity. Metabolic activity is a useful measure because it can help determine an organism’s temperature tolerances and provide an estimate for that organism’s optimal temperature where it has the best relative competitive advantage. The vast majority of animals on Earth (including *Eurytemora*) perform aerobic respiration, which means that they use oxygen to convert organic molecules such as sugars and fats into carbon dioxide, water, and energy (stored as the chemicals ATP, NADPH, or NADH). That energy is then used (i.e. those chemicals are broken down) to perform a wide variety of important functions including movement, growth, cellular maintenance, and reproduction. By measuring how much oxygen an organism consumes we can determine how much energy it is using at any given time.

To measure oxygen consumption, organisms are placed in a closed chamber and the oxygen content of the air or water that they are in is measured using an oxygen probe over a length of time. The respiration rate (in units of oxygen consumed per unit of time) is calculated by comparing the amount of oxygen that was present at the start of the experiment to the amount present at the end and dividing the difference by the duration of the experiment.

While measuring oxygen consumption is a relatively simple and robust method, there are some complications that can make the data hard to interpret and limit its applications to “real-world” scenarios that occur outside of the lab (Cammen et. al., 1990). First, the laboratory environment is not the same as the field. Differences in temperature, stimuli, light, movement, water chemistry, etc. that affect an organism’s activity may not be replicated *ex situ*. Second, organisms are starved before and during the experiment to eliminate confounding variables such as the respiration rate of the prey (which may result in a net increase in oxygen if the prey
photosynthesizes), or the bacterial decomposition of waste or decaying materials produced during feeding. Finally, respiration experiments have to be performed over a long enough period of time to obtain a measurable change in oxygen concentration using oxygen sensors. Moving organisms into and out of the closed chamber is stressful and may affect how animals initially react, so short-term experiments may provide an unreliable measure of oxygen consumption. Because of the unpredictability of the laboratory stressors and starvation, measured respiration rate may not provide an accurate estimate of an animal’s energy requirements in nature.

One alternative to direct respiration measurement is a biochemical assay that tests the activity of an organism’s metabolic enzymes. Enzymes are large biological molecules that catalyze reactions by making them more energy efficient. Many enzymes that are important in an organism’s metabolism are part of the Electron Transport System (ETS), in which electrons are removed from organic carbon (such as sugars) and given to O$_2$, the “terminal electron acceptor.” In this experiment, *Eurytemora* were homogenized in a buffer solution in order to lyse their cells and release the enzymes that are normally constrained in the interior mitochondria and microsomes. The enzymes in question remove electrons from the energy storage molecules NADPH and NADH and transfer them onto oxygen. However, in the presence of the chemical salt INT (iodonitrotetrazolium chloride), the enzymes work in the same manner but shuttle electrons to the chemical, which then changes color from a light yellow to a deep pink. The development of the pink color can be quantified using spectrophotometry and is a direct measure of how much INT was reduced by the enzymes. Since we know the duration of time the enzymes were active and the amount of product produced, we can then calculate the amount of activity in oxygen consumed per unit time; the same units as respiration.
Methods

Collection and Maintenance of Organisms

The experimental organisms used in this study were the calanoid copepod species *Eurytemora carolleae* (Alekseev & Souissi, 2011) and *Skistodiaptomus oregonensis* (Lilljeborg, 1889). A total of 1,326 individual copepods were used in the respiration and ETS experiments. Method-proofing and some preliminary tests of the ETS and respiration procedure were performed on other organisms including *Macrocyclops sp.*, *Acanthocyclops vernalis*, *Bythotrephes longimanus*, and *Ceriodaphnia*, spp. Animals were collected between July 25th and October 19th, 2018 in their natural assemblage from Little Sturgeon Bay (LSB) using a 0.5m-diameter plankton net with 250µm mesh. Previous work (Poli, 2004) found the highest concentration of *Eurytemora* in the shallow area surrounding a municipal boat dock, so that area was sampled. The net was towed by hand in duplicate for the length of the dock (~100m) and rinsed into a 5-gallon (18.9L) bucket using either aged tap water or water from LSB. Water from LSB was added so the total sample volume was approximately 4 gallons (15L). Any visible juvenile fish and large clumps of macrophytes that were collected from the sample were removed. The buckets were sealed with a lid and transported back to the lab. Live animals from LSB were identified and sorted manually under 10-40x magnification within 48 hours of transport back to the lab and placed into monospecific containers containing aged tap water. Dichotomous keys and illustrations from Balcer et. al. (1984) were used as morphological and taxonomic guides. Animals were handled using disposable, wide-mouthed plastic or glass pipettes to avoid injury.
Measurement of Respiration

The respiration rate of *E. carolleeae* and *S. oregonensis* was measured. The copepods used in respiration experiments were incubated in the dark for at least 48 hours at the given experimental temperature (9, 14, 18, and 22°C for *E. carolleeae* and 14° and 22° for *S. oregonensis*). They were provided the green algae *Scenedesmus*, which was determined by previous authors to be a good food source (Engstrom-Ost et al., 2017, Barrett 2014, Simčič and Brancelj 2004). After the incubation period, an average of 23 healthy, adult copepods were rinsed with aged tap water [oxygen-saturated, filtered through a GF/C filter (47mm diameter)] to remove any attached algae. The animals were then placed in 55mL glass chambers and sealed with an oxygen probe (Clarke-type micropulse sensor, Endeco, Inc.) secured into the bottle’s ground-glass opening. Respiration experiments included two or three experimental bottles (and two or one control bottles, respectively, containing aged tap water) that ran concurrently. All bottles were submerged in a 4L bucket that contained water at the incubation temperature. Experiments conducted at 9°, 14°, and 18°C were placed in an incubator in the dark while experiments run at 22°C were placed on the lab bench at the lab’s 22° room temperature underneath an opaque cover. Experiments were conducted for 12-24 hours and were terminated once the oxygen concentration in one experimental bottle reached 80% of the starting value. After each experiment, the respiration chambers were dried and cleaned with 95% ethanol to prevent bacterial growth.

Data was exported into Microsoft Excel, and the respiration rate was determined using a least-squares linear best fit regression over time. The average $R^2$ for all regressions was $>0.97$. Initial readings for all bottles often fluctuated (and even occasionally increased), so data that did not show a unidirectional trend were discarded. The respiration rate (oxygen consumption rate) $R$
was determined by subtracting the control slope (mgO$_2$/L/min.) from the experimental slope and transformed appropriately to obtain $R$ in the units µL O$_2$/animal/hour. Statistical tests were performed in PAST3. Non-parametric tests were used if assumptions of normality were not met.

**Determination of ETS activity**

ETS measurement procedure was determined following Simčič and Brancelj (2004) and Hernández-Léon (2000) but modified such that the homogenization and centrifugation steps were not performed between 0º and 4ºC. During these steps, refrigerated reagents were used, but the specific actions were performed at room temperature. Additionally, the Hernández-Léon (2000) procedure recommends diluting the homogenate to maintain activity within the sensitivity level of the experiment, but we found (in experiments described below) that this was not necessary for the activity found in this system.

In sum, the procedure had three principal steps:

1) Obtaining a cell-free homogenate: A group of healthy-looking copepods (average $N=23$; combined to constitute one “sample”) was rinsed in aged tap water to remove any attached algae and placed into Teflon glass grinders containing a homogenization buffer [0.1M sodium phosphate buffer pH 8.4, 75 mM MgSO$_4$, 0.15% (w/v) polyvinyl pyrrolidone, 0.2% (v/v) Triton-X- 100]. Organisms were manually crushed for 2 minutes, then transferred to a 4mL screw-top plastic centrifuge tube and placed on ice. The homogenate was then sonicated using a Branson ultrasonic tissue disrupter 20 seconds at 40W (20% amplitude) and centrifuged for 4 minutes at 10,000rpm.

2) Incubation: Each sample was prepared in triplicate or tetraplicate and incubated for 40 to 60 minutes at the experimental temperature along with a substrate blank and triplicate reagent
blanks. Each sample combined 0.5mL supernatant, 1.5mL substrate solution, and 0.5mL from a frozen stock of INT solution (2.5mM 2-p-iiodo-phenyl 3-p-nitrophenyl 5-phenyl tetrazolium chloride [Cayman Chemical Company] in milli-Q H2O). The substrate solution was prepared daily by combining stock buffer [0.1 M sodium phosphate buffer pH 8.4, 0.2% (v/v) Triton-X-100; kept frozen at -20ºC] with NADH and NADPH (Cayman Chemical Company) to achieve molar concentrations of 1.7 mM and 0.25 mM, respectively. The substrate blank (0.5mL supernatant and 1.5mL homogenization buffer) was prepared for each sample, and the triplicate reagent blanks (0.5mL homogenization buffer, 1.5mL substrate daily substrate buffer, and 0.5mL INT) were prepared for each incubation. Each incubation contained between 2 and 4 samples. After the incubation period, 0.5mL quench solution [equal parts formalin (36%, aqueous) and H3PO4 (concentrated)] was added to each sample and reagent blank to cease enzymatic activity and stop the abiotic reduction of INT.

3) Determination of potential oxygen consumption: The amount of INT-formazan was determined by spectrophotometry (Varian 50 Scan spectrophotometer) immediately after the addition of the quench solution. The absorbance was measured at 450nm, and the absorbance at 750nm was subtracted from that value as a turbidity blank. ETS activity (µL O2/hour) is calculated as follows from Hernández-Léon (2000):

\[
ETS = \frac{60 * H * AV * COD}{INT * T * L * F}
\]

\[
COD = \frac{(AOD * AV) - (BOD * BV) - (ROD * RV)}{AV}
\]

where \( H \) is the homogenization volume (4mL), \( AV \) is the assay volume (3mL), \( BV \) is the substrate blank volume (2mL), \( RV \) is the reagent blank volume (3mL), \( AOD \) is the difference between the absorbance of the assay at 450 and 750 nm (\( BOD \) and \( ROD \) are the same differences
for the substrate blank and reagent blank, respectively), INT is the conversion factor to volume of O\textsubscript{2} (1.42), \(T\) is the incubation time in minutes, \(L\) is the path length of the spectrophotometer cuvette (1cm), and \(F\) is the volume of homogenate used in each assay (0.5mL).

\textit{Determination of ETS:R ratios}

In order to obtain the most reliable comparison and to eliminate some of the potential variation in ETS activity between individuals, we also performed ETS assays on individuals that were used in the respiration measurement experiments. After each respiration experiment was completed, the copepods in that experiment were re-counted and washed with fresh, filtered aged tap water. Any copepods that had died in the respiration experiment or those that looked sickly were excluded from the subsequent ETS analysis. Excess water was removed using a paper filter, and the total wet weight of each batch of copepods was measured using a microbalance (Cahn C-35 ultra-microbalance, Thermo Electron Corp, Beverly, MA.). Animals were stored on pieces of aluminum foil at -20\textdegree C immediately after weighing. The ETS activity of those animals was determined as described above but were not re-rinsed with aged tap water. Instead, the frozen mass of copepods was removed from the foil added directly to the homogenization buffer in step one.

\textit{Method Proofing: Incubation time and Dilution}

In order to determine the effect of ETS experiment incubation time and determine if homogenate dilution would produce reliable results, we tested the effect of dramatically increasing the amount of ETS enzymes present. For this test we prepared triplicate assays of a comparable number (\(N = 16\)) of the much larger predatory zooplankton \textit{Bythotrephes longimanus}. The absorbance at 450 and 750 of each of the assays and a reagent blank was
measured every minute for one hour, and again at 76 and 105 minutes. The ETS value was calculated for each observation of each replicate for the duration of the experiment.

*Determination of the Arrhenius Activation energy (Eₐ)*

The results of the ETS:R ratio experiments were used to determine the Arrhenius activation energy (Eₐ; expressed in kcal/mole) for *Eurytemora carolleeae* according to the calculations in Packard et. al. (1975). The Eₐ relationship can be expressed as:

\[ Eₐ = -RS \]

where R is the gas constant (1.987 kcal/mole) and S is the slope of a linear least-squares regression. An Arrhenius plot with a linear least-squares regression was created in Microsoft Excel that graphed the natural log (ln) of ETS activity against the reciprocal of absolute temperature (°K). The ETS value used for each temperature was the average of the replicate ETS activities used to determine the ETS:R ratio at that temperature. 95% confidence intervals of the slope (and by consequence the 95% confidence intervals [95% CI] for Eₐ) were determined using a bivariate ordinary least-squares regression in PAST3.

**Results**

*Respiration Rates*

The measured respiration rate of *E. carolleeae* increased from 0.025 ± 0.011 µL O₂/animal/hr at 9°C (mean ± 95% CI, n= 4) to 0.238 ± 0.088 µL/animal/hr at 26°C (n = 3) (Fig. 1). The data can be best modeled by the linear least-squares regression \( R = 0.0128 * \) *Temperature[°C] – 0.0897 (R²=0.996; p = 0.007)*. The respiration rates of *S. oregonensis* at the two temperatures measured did not differ significantly, with a value of 0.083± 0.035 µL O₂/animal/hr at 14° (n = 3) and 0.052± 0.027 µL O₂/animal/hr at 22°C (n = 2; t-test, p>0.3). The
Respiration rate of *S. oregonensis* was not significantly different from that of *E. carolleeae* at 14°C (t-test; p>0.8) but was significantly lower at 22°C (t-test; p<0.05).

**Figure 1.** Measured respiration rate of *E. carolleeae* and *S. oregonensis* at different temperatures. Individuals were acclimated to the experimental temperature for at least 48 hours before the start of the experiment. Error bars represent 95% confidence intervals.

**Effect of the respiration experiment on ETS activity**

To determine the effect (if any) of the respiration experiment on calculated ETS activity in *E. carolleeae*, the ETS activity of animals used in respiration experiments was compared to those animals that had not been used in respiration experiments (Fig. 2). There was no significant difference between groups at either temperature (t-tests; both p>0.05).
Calculated ETS activity in *E. carolleeae* for batches of individuals (*N* = 23 individuals per sample) that were used in respiration experiments (*x*° Resp) compared to those not used in respiration experiments (*x*° No Resp) at 18° and 22°C. Error bars represent 95% confidence intervals (sample *n* > 4).

**Incubation time and dilution**

To determine if homogenate dilution was necessary (i.e. if the ETS enzymes consumed enough NADH, NADPH, or INT to become unsaturated during the course of the experiment), we drastically increased the amount of ETS enzymes above any reasonable experimental value for 20-30 copepods and allowed the experiment to progress for much longer than suggested by other authors. The calculated COD (corrected optical density) increased with time (Fig. 3) and can be most accurately described by a linear least-squares regression (*R*²=0.9995). The calculated ETS value (*µLO₂/animal/hr*) decreased drastically during the first 17 minutes of the assay but reached an asymptote after 17 minutes (Fig. 4). This was not due to a change in the rate of increase of COD, but the natural stabilization built into the ETS calculation equation where only COD and *T* are changing and do so in a linear fashion.
ETS activity of *E. carolleeae*

The calculated *in situ* ETS activity of *E. carolleeae* increased with increasing temperature until reaching a peak around 22°C (Fig. 5). ETS was the lowest at 9°C with a value of 0.031 ± 0.014 µL O₂/animal/hr (mean ± 95% CI, n = 4) at 9°C and increased to its maximum of 0.076 ± 0.007 µL O₂/animal/hr at 22°C (n = 4). ETS activity subsequently decreased to 0.053 ± 0.014 µL O₂/animal/hr at 26°C (n = 3). The relationship between average ETS activity and temperature from 9° to 22° can be best explained by the exponential least-squares regression: \( ETS = 0.0162 \times e^{6.57 \times 10^{-2} \times Temp[°C]} \) (R²>0.97).
**Figure 4.** Calculated ETS activity over time using the calculated COD presented in Fig. 3. Values represent the average of three replicate runs from the same sample homogenate. Error bars represent 95% confidence intervals.

**Figure 5.** Calculated ETS activity (µL O₂/animal/hr) of *E. carolleae*. Animals were acclimated for at least 60 hours at the incubation temperature before measurement. ETS incubation was performed at the acclimation temperatures. Values represent the average of replicate samples ($3 \leq n \leq 7$). Error bars represent 95% confidence intervals.
Calculation of the ETS:Respiration Ratio

The ETS:R ratio for animals acclimated to the respiration and incubation temperatures decreased with increasing temperature (Fig. 6). The calculated ratios (Temp[ºC] = mean ± 95% CI) for *E. carolleeae* are as follows: 9ºC = 1.28 ± 0.70 (n = 4); 14ºC = 0.45 ± 0.22 (n = 6); 18ºC = 0.37 ± 0.11 (n = 7); 22ºC = 0.38 ± 0.07 (n = 4); 26ºC = 0.22 ± 0.09 (n = 3).

![Figure 6](image_url)

**Figure 6.** ETS : Respiration ratio (Mean ± 95% CI) for *E. carolleeae* at 9º, 14º, 18º, 22º, and 26ºC. ETS activity was measured directly after each respiration experiment. Animals were acclimated to the experimental temperature for at least 48 hours before the respiration experiment, and ETS assays were incubated at the respiration temperature.

Arrhenius Activation Energy (Eₐ)

The Arrhenius activation energy (*Eₐ*; expressed in kcal/mole) for *Eurytemora carolleeae* was calculated using the slope of a linear least-squares regression line of the natural log of ETS activity and the inverse of the absolute temperature (ºK) (Fig. 7). ETS data from 26ºC was excluded as it was past the maximum level of enzyme activity and therefore does not accurately
reflect the temperature dependence on enzyme speed. The $E_A$ for *E. carolleae* was found to be $11.1 \pm 3.7$ kcal/mole.

**Figure 7.** Arrhenius plot showing the ln (ETS activity) against the reciprocal of absolute temperature for *E. carolleae*. All ETS assays were incubated at the copepods’ acclimation temperatures (9ºC, 14ºC, 18ºC, and 22ºC). Values for ETS activity represent the average of all ETS assays performed at that temperature. The dashed line is the least-squares linear regression of the data ($R^2=0.97$).

**Discussion**

**Points of Emphasis**

This study’s primary concern is the metabolic activity of the invasive calanoid copepod *Eurytemora carolleae*. Metabolic activity was quantified using traditional respirometry and a modified version of the Electron Transport System (ETS) assay (Owens & King, 1975) as described by Hernández-Léon (2000) and Simčič and Brancelj (2004). We measured the respiration rates of *E. carolleae* and determined its *in-situ* ETS activity using acclimated
animals over a range of temperatures from 9º to 26ºC. From these measurements, we determined the Arrhenius activation energy $E_A$ for the copepod’s metabolic enzymes. Several method-proofing and control experiments were performed as robustness checks on the ETS procedure to determine significant sources of error, optimize it for the system of interest, and to test the reliability of ETS activity as a proxy in-situ respiration. A long-term goal of this research is to assess how anthropogenic perturbations to the Green Bay – Little Sturgeon Bay system may affect the success of *E. carolleae* in relation to its native competitors.

**Interpretation of Results: Respiration Rate**

Respiration rates for *E. carolleae* and *S. oregonensis* in this experiment compare well with the range of values in previously published studies. In their 1999 study, Adrian et. al. measured respiration rates of marine Baltic *E. affinis* that ranged from 0.04 µL O$_2$/animal/hr at 6ºC and 0.3 µL O$_2$/animal/hr at 18ºC. A summary of respiration data in Lampert (1984) exhibited respiration rates between 0.01 and 0.22 µL O$_2$/animal/hr for several copepod species at temperatures ranging from 15º to 20ºC. However, most species in that temperature range had measured respiration rates between 0.05 and 0.1. Values greater than 0.1 were observed in larger species such as *Aglao diaptomus leptopus* and *Limnocalanus macrurus*. Richman (1958, in Lampert 1984) measured the respiration rate of *S. oregonensis* to be 0.090 µL O$_2$/animal/hr at 22ºC, which is well within the confidence intervals of our data at that temperature.

The respiration rate of *Eurytemora* species presented in this study and reported by Adrian et. al. (1999) are consistent, but they are higher than most other species, especially at higher temperatures. This may be explained in part by the organisms’ life history and recent invasion into fresh water from its native brackish habitat (Mills, 1993 and Kipp, 2006). Lee et. al. (2013) found that food abundance was an important determinant of the survival rate of North American
*Eurytemora* coping with low-salinity environment. They found that survival rate was significantly higher when there was a large stock of available food, as the copepod could offset the metabolic cost of maintaining homeostasis in a low-ion environment. The authors also propose that this phenomenon will occur until the established population can adapt given sufficient evolutionarily time. In this context and because the invasion of *Eurytemora* into the Great Lakes is recent on an evolutionary timescale, the high respiration rates observed in this study are understandable.

*Interpretation of Results: ETS activity and ETS:R Ratios*

When discussing the relationship between ETS and respiration, there is some inconsistency in the literature. Some authors provide data as R:ETS (e.g. Cammen et. al., 1990, King & Packard 1975) and others provide data as ETS:R (e.g. Owens & King, 1975). For ease of discussion, all data from other authors presented here have been transformed to ETS:R. In this study, we found that the ETS:R for *E. carolleaeae* ranged from 0.22 to 1.28 (Fig. 6). These values are considerably lower than the reported values of both marine and freshwater zooplankton from other authors. Owens and King (1975) found a ratio of 2.02 for the predatory copepod *Calanus pacificus*. Packard (1985) and Rai (2002) cite 2.04 and 1.96, respectively as the appropriate ETS:R ratios for zooplankton. Simčič and Brancelj (2004) found ETS:R ratios of approximately 2 in several *Daphnia* hybrids. Early data from King and Packard (1975) appears to find ETS:R values of between 0.46 to 0.78 for marine copepods. However, Devol and Packard (1978) posit that this data is not comparable to the data presented in our study as the King and Packard (1975) ETS assay did not include Triton-X-100 as a detergent. After transforming the data from King and Packard (1975) using an empirical coefficient to account for the lack of Triton-X-100, Devol
and Packard (1978) find that the bulk ETS:R ratio for that dataset was found to be 1.96 as well. There are several possible explanations that could explain the difference between these findings and the data in this study that will be explored presently:

1) Acclimation to the lab or drastic changes due to respiration experiment: Many authors have noted rapid decreases in respiration rate in organisms once transferred to the lab without a proportional decrease in ETS activity for many days (Ikeda, 1977; Kiørboe et al., 1985; Cammen, 1990). That means that our ETS:R ratio is likely an overestimate of the in-situ value in LSB. Therefore, the difference in ETS:R ratio between this study and the literature consensus is most likely caused by lower-than-expected ETS activity, not higher-than-expected respiration. For example, Båmstedt (1980) found the ETS activity for *Acartia tonsa* to be between 0.12 and 0.14 µL O₂/animal/hr. ETS activities in this study only reached half of that value with a maximum rate of 0.076 µL O₂/animal/hr at 22°C (Fig. 5). Other work has stressed the important effect of physical stress from capture and fed versus starved conditions on metabolic activity (e.g. Ikeda, 1977; Båmstedt, 1980; Cammen 1990). While those studies suggest that we indeed are underestimating the in-situ respiration rate given our measurement procedure, any immediate or drastic change in respiration rate after transport to the lab would be missed due to the incubation of the animals at the desired temperature for two days. The effect of starvation on the respiration rate should be negligible, as incongruous or extremely variable data at the beginning of each respiration experiment were discarded and so the metabolic adjustment from fed to starved conditions or changes in behavior associated with that transition time would be eliminated as well. To examine the effects that starvation during respiration had on the measured ETS, we directly compared the enzymatic activities of animals used in respiration experiments against those that were not (Fig. 2). We found no significant difference between groups, which
makes sense in the context of previous work and given the relatively short duration of starvation. In sum, any lab-caused alterations to the metabolic activity of *E. carolleae* in this study either underestimate the respiration rate (and therefore overestimate the ETS:R ratio) or are on a timescale that has been identified by previous work and confirmed experimentally in this study to not have a significant effect on ETS enzymatic activity.

2) Hernández-Léon, (2000) stresses the importance of sterile technique when preparing the INT samples to avoid bacterial contamination. Although cross contamination of samples was avoided, completely sterile technique including sterile extraction of reagents and sterile glassware was not employed in this experiment. Despite this, I do not believe that bacterial contamination was responsible for skewing the results. The only reagent that would support significant bacterial growth was the substrate buffer (NADH + NADPH solution), which was produced daily just before the experiment, giving very little possible bacterial replication time. Furthermore, if bacteria were present, because the same substrate solution was used in the reagent blanks and the sample assays, their effect on INT reduction would be subtracted away during the calculation of the COD, leaving only the effect of the ETS enzymes. If bacteria bloomed in the sample assay vials after the homogenization of the copepods (which is a more likely scenario), we would expect to find higher-than-expected ETS activities as the bacterial metabolic enzymes would contribute to the formation of INT-Formazan. Bacterial growth during respiration experiments would lead to higher-than-expected respiration rates but consistency with other studies makes a systematically elevated respiration measurement due to bacteria unlikely.

3) The effect of assay conditions on ETS has been well-characterized (e.g. Owens & King, 1975; Båmstedt, 1980). The most common area of concern is the duration of sample storage between death and ETS analysis. There is conflicting data on this topic and is worth
investigation. Båmstedt (1980) suggests that storage of deceased animals at -20°C (as was done in this study) results in a very rapid and precipitous drop in ETS activity, while others (Ahmed et. al. 1976, in Hernández-Léon, 2000) found no decrease in activity after nearly a month. Despite this conflict, there is a general consensus that the amount of time enzymes reside in cell-free homogenate solutions should be minimized and that spectrophotometry should be performed as soon as possible. None of these storage problems are likely to have affected this study’s data, since storage times at all points were much shorter than the problematic durations enumerated above. In most cases, and especially for the data used to determine the ETS:R ratios, ETS activities were measured within hours if not immediately after the end of each respiration experiment. Furthermore, if there was an extreme and rapid decrease in ETS activity while frozen, we would see a disparity in organisms frozen overnight as compared to those that were immediately analyzed. Such a decrease was not observed in this experiment. Homogenate was never stored for more than two hours and was always kept on ice or in a 4°C refrigerator. Additionally, the results of the time-series data at 22°C (Fig. 3) cast doubt on the impact of homogenate time, as there was no decrease in ETS activity up to two hours after the start of the experiment. If the enzymes were rapidly degrading at room temperature, we would expect to see the COD’s rate of increase to slow accordingly. As this was not observed, it follows that homogenate degradation was not a significant complicating factor within the timescale of these ETS assays.

4) Deviation from $V_{\text{max}}$. Many authors discuss diluting the homogenate as an essential step in the ETS methodology as the assay would lose sensitivity if the enzymes were not always at $V_{\text{max}}$. If the creation of INT-formazan by enzyme-mediated reduction was enough to significantly change the INT, NADPH, or NADH concentration such that any reactant became
limiting, we would expect the INT-formazan production rate to decrease over time. That change would present itself in a plot of COD vs time as a shallowing exponential curve. Such a scenario was not observed in this study, and a linear relationship between COD and time was observed even when ETS enzyme activity was upwards of 14x higher and 2.5x longer than in experiments with *E. carolleeae* homogenate (Fig. 3).

5) The importance of incubation time. The optimal incubation time recommended for ETS assays varies by author from 10 minutes (Rai, 2002) to 20 minutes (Hernández-Léon, 2000 and Owens & King, 1975) to 40 minutes (Simčič & Brancelj, 2004). The time-series data presented in Fig. 4 directly contradict the 10-minute optimum and suggest that incubation time is a negligible factor in the final ETS activity calculation past 17 minutes. In fact, longer incubation times such as those suggested by Simčič and Brancelj (2004) would make errors in timing less important overall as they would constitute a smaller proportional difference in the final data calculations.

6) The most logical human error that would affect all experiments equally and result in depressed ETS activity would be an improper preparation of the stock substrate solution in which the detergent Triton-X-100 was omitted. It is suspicious that the ETS data and ETS:R ratios are different from the published consensus but are very comparable to the iteration of the ETS from King and Packard (1975) that did not include Triton-X-100.

7) The ETS:R ratios less than one found for this population are not consistent with the existing literature. Packard (1985) explains how potential enzymatic activity (the Vmax of aerobic metabolism presented as ETS activity) should always be less than the resting respiration rate. Because ETS activity responds more slowly to environmental changes, it must always be maintained at a higher level such that in the event that a higher metabolic rate is required (to
quickly reproduce, escape a predator, hunt, migrate, etc.) there is not a time delay as the animal produces more metabolic enzymes. A 1:1 ETS:R ratio would then indicate that the organism is operating at its maximum metabolic rate (VO₂ max) and could not increase respiration any further without a lengthy acclimation period in which more enzymes are produced de novo (Packard, 1985). Therefore, a ratio less than one would seem impossible under the theoretical guidelines. But, as there are no obvious methodological explanations (outside of critical human error in the preparation of reagents) that can explain the consistently low ETS and ETS:R values determined in this study, it is possible that the physiology of *E. carollaeae* differs in some fundamental way from other zooplankton species. The studies previously cited include ETS data from zooplankton within their natural habitat. Perhaps the environmental disequilibrium of an osmotically stressed, recent freshwater invader such as *E. carollaeae* causes a different physiological response than those species in their native habitats. Or, maybe this constant stress results in a different equilibrium physiological condition in which the enzymes become impaired when removed from the homeostatic in vivo environment during homogenization.

*The Arrhenius activation energy (Eₐ)*

Despite the differing magnitudes of the measured ETS values of *E. carollaeae*, the Arrhenius plot presented in Fig. 7 closely mirrors relationship between activity and temperature as described by Packard et. al. (1975). The data in this study display the characteristic exponential increase in enzymatic reaction rates followed by a sharp decrease after some temperature maximum (see Packard et. al., 1975 and Simčič & Brancelj, 2004 for comparison). The thermal maximum observed in *E. carollaeae* around 22°C was lower than the reported maximum temperatures near 40°C for copepod assemblages as reported by Packard (1975) but were close to the 25°C inflection point determined by Simčič and Brancelj (2004). The
inconsistency in thermal maximum illustrates the importance of species identity on ideal enzymatic kinetics.

The calculated Arrhenius activation energy $E_A$ of 11.37± 3.7 kcal/mole is also comparable to other authors. Other authors (e.g. Packard et. al., 1975 and Simčič & Brancelj, 2004) have found $E_A$s for other planktonic organisms or assemblages that compare favorably to the calculated value for *E. carolleeae* presented in this study. Packard and King (1975) determined an $E_A$ of 15.2 kcal/mole for a copepod assemblage and reported values between 11.7 and 21.9 kcal/mole for other bulk plankton assemblages. An $E_A$ of 15 kcal/mole was used in subsequent experiments (e.g. King and Packard (1975); Devol and Packard 1978). Simčič and Brancelj (2004) recorded $E_A$ values between 11.04 and 17.76 for hybrid *Daphnia* populations and Pascal and Chong (2016) reported an $E_A$ of 8.3 to 18.2 for the copepod species *Pseudodiaptomus annandalei*. Generally, *E. carolleeae* has a lower $E_A$ and enzymatic thermal maximum than other species. But, given *E. carolleeae* is native to colder waters (Balcer et. al., 1984) and is most common during colder parts of the year in the Great Lakes system (Torke, 2001), that result is not surprising. Enzymes with lower activation energies, while unstable at higher temperatures, are more efficient at the lower temperatures to which *E. carolleeae* evolved.

**Conclusions and Implications**

This study has several implications for both the utility of the ETS assay as a proxy measurement of respiration rate and for the future success of *E. carolleeae* in Little Sturgeon Bay, WI. The respiration rate of *E. carolleeae* was higher than its native competitor *S. oregonensis* at temperatures above 14°C, and all respiration rates are within the ranges presented in other studies. The Arrhenius activation energy also compares favorably to other zooplankton.
ETS data suggests that the thermal limit of *E. carolleeae* is around 22ºC, which supports previous findings. Although the ETS values and the ETS:R rates determined in this experiment differ considerably from previously published values, robustness checks in this procedure eliminate common sources of reduced enzymatic activity, principally activity lost during storage or during starvation, that were well characterized by other authors. Because *E. carolleeae* is native to and most common in colder, brackish environments, the warming trends associated with climate change may make the copepod less competitive in warm, fresh water due to higher ambient levels of stress. However, due to the copepod’s ability to consume the toxic cyanobacteria that are associated with eutrophication, those deficits and extra metabolic expenditures may be overcome.

**References Cited**


