A METHOD FOR MEASURING IN SITU OXYGEN CONSUMPTION RATES OF FRESHWATER GASTROPODS

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A method for measuring in situ oxygen consumption rates of freshwater gastropods

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Abstract. In situ oxygen consumption rates can be used to evaluate respiratory costs of organisms in the natural environment. We determined the in situ oxygen consumption rates of freshwater gastropods by using an assay of the coenzymes involved in the electron transport system (ETS). This assay may be used to estimate in situ and in vitro metabolic rates of individuals when direct monitoring of oxygen consumption is not practical. The relationship between ETS activity and measured oxygen consumption was determined for individuals of 3 common Lake Michigan snail species: Elimia litescens, Stagnicola unovalrifl, and Physella integra. The snails were exposed to various temperature conditions to stimulate a wide range of oxygen consumption. After 14 d of acclimation to the experimental conditions, oxygen consumption rates were measured daily. After 5 d, the snail tissue was removed from the shells, flash frozen, and stored in liquid nitrogen until the ETS assay could be performed. Oxygen consumption was highly correlated with ETS activity for all 3 species (p < 0.001). The in situ oxygen consumption rates of these snails can be evaluated using the calibration of oxygen consumption to ETS activity.

Keywords: electron transport system (ETS), gastropod, oxygen consumption.

Accurate measurement of in situ oxygen consumption in aquatic invertebrates is difficult, especially for gastropods, but is often critical for addressing questions about the respiratory costs of seasonal changes (Bayne et al. 1977), migration (Schneider and Lyons 1993), locomotion (Innes and Houlihan 1985), exposure to environmental toxicity (Chapman and Connell 1986, Bharathi and Prasada 1989, Sivaramakrishna et al. 1991), predator avoidance (Carefoot and Donovan 1995), and fouling by organisms such as the zebra mussel (Mackie 1991). Previous gastropod studies have relied on in vitro studies of metabolic activity, combined with formulas (Åkerlund 1969, Fitch 1975), for estimating allometric and temperature effects on in situ oxygen consumption rates (Thornton and Lessem 1978). However, estimates of oxygen consumption based on controlled laboratory conditions may not accurately reflect actual oxygen consumption rates in the field.

The estimation of oxygen consumption rates from body mass and temperature alone could bias results because of interference from environmental and physiological factors such as habitat variation in oxygen concentration (Berg and Ockelmann 1959, Åkerlund 1974), food availability (Newell and Roy 1973), and variable feeding rates and activity levels (Halcrow and Boyd 1967, Newell and Roy 1973). Chamber effects (Bayne et al. 1977) and fouling by other organisms (Mackie 1991) can also make direct measurement of oxygen consumption impractical or impossible.

We tested a method for accurately determining in situ oxygen consumption rates that circumvents these problems. In most organisms, oxygen is metabolized through the electron transport system (ETS) (Klingenberq 1968). Measuring the activity of this system via the ETS assay (Owens and King 1975, Packard 1985) provides an estimate of individual average in situ oxygen consumption over the previous 7-21 d. The method has been used to estimate
such as contaminant concentrations in tissues convincingly demonstrates that exposure has occurred. These 5 or 6 elements represent the minimal requirements for a credible causal argument that integrates results of multiple studies and demonstrates concordance of laboratory and field results.

**Example application of assembly rules**

Beyers et al. (1995) studied the response of aquatic invertebrates in the Little Missouri River, North Dakota, to an aerial application of the insecticide Sevin-4-Oil (40.5% carbaryl active ingredient) on surrounding rangeland. Invertebrate drift was quantified by deploying 3 drift nets across the channel at reference and impact sites. The reference site was located ~36 river-km upstream of the impact site. Nets were collected continuously for 4 consecutive 3-h periods from 0700 to 1900, 2 d before and 2 d after pesticide application. Depth-integrated water samples were collected for pesticide analysis at reference and impact sites. Inspection of data from this study led to the conclusion that aquatic invertebrates were affected on the day of pesticide application (Fig. 2). Application of the assembly rules explicitly identified why the re-
response was intuitively obvious and integrated supporting data from other sources.

There was a strong association between pesticide application and increased invertebrate drift. Invertebrate drift increased in all 3 samples collected at the impact site during the 3-h period immediately following pesticide application, whereas none of the reference samples showed this response. Strength of association is also supported by good agreement between drift-net collections within each sampling interval at each site, and pairing of reference and impact sites. There is a consistent association between pesticide exposure and increased drift. Several investigators have observed increased drift after field applications of Sevin-4-Oil to upland areas surrounding streams (citations omitted for brevity; see Beyers et al. 1995). Specificity of effect contributes little to this particular causal argument because invertebrate drift can be influenced by a variety of factors. The lack of specificity is demonstrated by the data for 6 July when localized rain showers changed river conditions and invertebrate drift. In contrast, temporality is a critical component of this causal argument. Most significant is the co-occurrence of maximum pesticide concentration and increased invertebrate drift immediately after application. If the increase in invertebrate drift had occurred before pesticide application, it would have cast serious doubt on the causal argument. The invertebrate response was also consistent with a biological gradient. On the day of application, the magnitude of drift was greatest when carbaryl concentration was high, and it declined with declining ambient concentration. Subsequent sampling showed that the decline was not a consequence of complete elimination of the invertebrate fauna, because organisms at both impact and reference sites were captured at similar rates. Several biologically plausible mechanisms may explain pesticide-induced invertebrate drift, including mortality, behavioral changes that increase the likelihood that a living invertebrate is swept into the water column, and passive defense to avoid prolonged exposure to an irritant. Most potential mechanisms have been investigated under laboratory conditions and constitute the bulk of the experimental data that support the causal argument. Other experiments have described lethal and sublethal toxicity. Analogous results have been obtained in both field and laboratory with other carbamate, organophosphate, and organochlorine insecticides. The former 2 classes of insecticides have the same mode of action (acetylcholinesterase inhibition) as Sevin-4-Oil. Indicators of exposure that could have contributed to the causal argument include detecting pesticide concentrations or enzyme inhibition in drifting organisms. These data were not collected, but other studies have shown that the active ingredient in Sevin-4-Oil can be detected in aquatic insects within 0.5 h of exposure; consequently, it is likely that pesticide concentrations were elevated in drifting organisms. All of the evidence is coherent. Laboratory experiments have demonstrated that lethal concentrations for many aquatic insects are less than concentrations observed in the Little Missouri River. Closely related taxa have been studied in the field and laboratory and have shown responses under exposure conditions. Last, no negative evidence was uncovered to suggest that the observed response was caused by some factor other than pesticide application.

Conclusion

Investigators should be aware of the inherent difficulties of using test statistics to infer causation based on unreplicated studies. Failure to meet 2 basic assumptions of inferential statistics invalidates this approach for demonstrating causation. This failure does not imply that experimental designs and statistical procedures for impact studies are not valuable. On the contrary, the methods are extremely valuable because they contribute to an ability to detect differences in response variables at impact and reference sites. These statistical procedures and designs allow quantitative demonstration that things changed after the impact occurred. The demonstrations are critical pieces of evidence for causal arguments.

Explicit use of assembly rules for making causal arguments allows investigators to efficiently organize, study, and present available evidence. This approach should be the basis for causal inference in environmental impact studies. The practical consequences of making a case for causation based on logical argument rather than statistical tests will probably be relatively minor. It is likely that past use of statistical tests resulted in scientific conclusions being right, but for the wrong reasons. In contrast, the philo-
test tube into the water bath, allowed the temperature to stabilize for 1 min, stirred the contents of the test tube briefly by hand, and then poured the solution into a cuvette. We allowed the solution in the cuvette to stabilize in the spectrophotometer for the first 5 min of the reaction. ETS activity was calculated from the slope of the change in absorbance between 5 and 10 min and the average temperature during this period. We converted enzyme activity into mg of oxygen consumption for each snail using the formula (Owens and King 1975):

$$\text{ETS}_k = \frac{\text{AVH}}{\text{SM}} \times K$$  \hspace{1cm} [1]

where $\text{ETS}_k$ is enzyme activity (mg O$_2$ g tissue$^{-1}$ h$^{-1}$), $A$ is the slope of the change in absorbance over time (absorbance cm$^{-1}$ min$^{-1}$), $V$ is the ETS enzyme assay volume (mL), $H$ is the volume of tissue homogenate (L), $S$ is the volume of cell-free extract (mL), $M$ is the wet mass of homogenized soft bodies (g), and $K$ is a unit conversion factor that equals 60.377. We also calculated enzyme activity (mg O$_2$ individual$^{-1}$ h$^{-1}$) using the formula:

$$\text{ETS}_i = \text{ETS}_k \times M$$  \hspace{1cm} [2]

Differences in temperature between acclimation and the ETS assay solutions were minimal ($\pm 1^\circ$C), but they were accounted for by the formula (Owens and King 1975):

$$\text{ETS}_{\text{adjusted}} = \text{ETS}_k \times \left[\frac{1}{E} - \frac{1}{R}\right]/7549.07$$  \hspace{1cm} [3]

where $E$ is the mean temperature (K) of the ETS assay solution during the time absorbance was recorded in the spectrophotometer, and $R$ is the mean ambient temperature (K) at which oxygen consumption rates were measured.

**Data analysis**

Data were analyzed using SYSTAT 5.0 for DOS (SPSS Inc., Chicago, Illinois) unless otherwise indicated. For each species we used least squares linear regression analysis to determine the correlation between standardized oxygen consumption rate (mg O$_2$/h) vs ETS$_k$ (mg O$_2$ g$^{-1}$ h$^{-1}$), and oxygen consumption rate per individual (mg O$_2$ ind$^{-1}$ h$^{-1}$) vs ETS$_i$ (mg O$_2$ ind$^{-1}$ h$^{-1}$). We used the following formula to standardize oxygen consumption rates (Bayne and Newell 1983):

$$V_{O_2e} = [W_s/W_e]^b \times V_{O_2e}$$  \hspace{1cm} [4]

where $V_{O_2e}$ is the oxygen consumption rate of a standard size animal, $W_s$ is the average mass of the snail species, $W_e$ is the mass of the experimental snail, and $V_{O_2e}$ is the oxygen consumption rate per individual for the experimental snail. We used 0.64 for the exponent b, the average for all marine grazing snails reported by Bayne and Newell (1983). The average mass used for each species to standardize oxygen consumption rates was 82.0 mg for *E. lividescens*, 56.3 mg for *P. integra*, and 60.3 mg for *S. woodruffii*.

We also evaluated the relationship between oxygen consumption, temperature, and wet mass using least squares regression analysis. If either temperature or wet mass explained more of the variation in oxygen consumption rates than the ETS assay, they would be a better predictor of individual oxygen consumption, and the ETS assay would be less valuable as a tool for predicting oxygen consumption rates.

To test for bias we examined whether the ratio of oxygen consumption per individual/ETS$_i$ (R/E) varied with either temperature (°C) or body mass (mg) for each species. The range of values of the R/E ratio should not change in relationship to either temperature or body mass. We used linear regression analysis to determine if the R/E ratio was dependent on either wet body mass or temperature.

**Independent tests**

We evaluated the predictability of the correlation between oxygen consumption (mg O$_2$ ind$^{-1}$ h$^{-1}$) and ETS$_i$ (mg O$_2$ ind$^{-1}$ h$^{-1}$) using independent tests. We followed the same procedures as described for measuring oxygen consumption rates and the ETS assay. We then calculated a predicted oxygen consumption rate for each snail using the formulas generated by the specific linear regressions described above. Graphs of measured oxygen consumption rates vs the predicted oxygen consumption rates should yield a 1:1 relationship. We tested the predictability of the calibrated regression equation by observing the deviation of the line generated by independent points from the 1:1 line. We tested for significant deviation from the slope of the 1:1 line using a $t$-test (Zar 1984).
Table 1. Equations of the predictive linear regressions of (A) oxygen consumption (mg O$_2$ ind$^{-1}$ h$^{-1}$) vs ETS$_i$ (mg O$_2$ ind$^{-1}$ h$^{-1}$), and (B) standardized oxygen consumption (mg O$_2$/h) vs ETS$_i$ (mg O$_2$ g$^{-1}$ h$^{-1}$) for 3 species of Lake Michigan gastropods.

<table>
<thead>
<tr>
<th>Species</th>
<th>Equation</th>
<th>$r^2$</th>
<th>$p$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elimia livescens</td>
<td>Oxygen consumption = 0.005 + 0.038 (ETS$_i$)</td>
<td>0.770</td>
<td>&lt;0.001</td>
<td>26</td>
</tr>
<tr>
<td>Physella integra</td>
<td>Oxygen consumption = 0.005 + 0.037 (ETS$_i$)</td>
<td>0.585</td>
<td>&lt;0.001</td>
<td>19</td>
</tr>
<tr>
<td>Stagnicola woodruffi</td>
<td>Oxygen consumption = 0.003 + 0.070 (ETS$_i$)</td>
<td>0.742</td>
<td>&lt;0.001</td>
<td>24</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. livescens</td>
<td>Oxygen consumption = 0.006 + 0.003 (ETS$_i$)</td>
<td>0.484</td>
<td>&lt;0.001</td>
<td>26</td>
</tr>
<tr>
<td>P. integra</td>
<td>Oxygen consumption = 0.006 + 0.002 (ETS$_i$)</td>
<td>0.250</td>
<td>&lt;0.001</td>
<td>19</td>
</tr>
<tr>
<td>S. woodruffi</td>
<td>Oxygen consumption = 0.003 + 0.004 (ETS$_i$)</td>
<td>0.589</td>
<td>&lt;0.001</td>
<td>24</td>
</tr>
</tbody>
</table>

Field measurements

We collected E. livescens from southwestern Lake Michigan (9.5 m, 12.8°C) to use the ETS assay to predict in situ oxygen consumption for this species. We used the same analytical procedures for these field-collected animals as described above for setting the predictive linear regressions.

Results

Our comparisons of the regressions for each species showed that both standardized oxygen consumption vs ETS$_i$ (Table 1), and oxygen consumption per individual vs ETS$_i$ (Fig. 2 and Table 1) were significantly correlated ($p < 0.001$). However, in all cases oxygen consumption vs ETS$_i$ exhibited a stronger linear relationship than oxygen consumption vs ETS$_o$. Therefore, we used ETS$_i$ as a predictor of in situ oxygen consumption rates in the 3 species tested. Results of measured oxygen consumption using our method were consistent with oxygen consumption rates of other freshwater gastropods (Berg and Ockelmann 1959).

Both temperature and wet-body mass were significantly correlated with oxygen consumption rates per individual (Table 2). However, these relationships always had lower correlation coefficients ($r^2$) than the ETS$_i$-oxygen consumption relationship.

We found no significant change in the range of values of the R/E ratio over a range of either wet body masses or temperatures (Fig. 3, 4).

Independent tests

For all 3 species the slope of the lines generated by the independent points did not differ significantly from the expected 1:1 line (Fig. 5) (E. livescens: $\beta = 0.940$, SE = 0.501, $p > 0.5$; P. integra: $\beta = 1.499$, SE = 0.652, $0.5 > p > 0.2$; S. woodruffi: $\beta = 0.871$, SE = 0.226, $p > 0.5$). We found a significant correlation ($p < 0.05$) between measured oxygen consumption and predicted oxygen consumption for S. woodruffi ($r^2 = 0.679$, $n = 9$) and P. integra ($r^2 = 0.370$, $n = 11$). However, for E. livescens the correlation between measured oxygen consumption and predicted oxygen consumption was low ($p = 0.09$, $r^2 = 0.261$, $n = 12$).

Field measurements

We calculated oxygen consumption rates for the field collected E. livescens using the predictive regression formula for this organism (Fig. 2). In situ oxygen consumption rates for this species fell well within the range of oxygen consumption recorded in the laboratory (0.008–0.018 mg O$_2$ ind$^{-1}$ h$^{-1}$, $n = 17$).

Discussion

This study shows that the ETS enzyme assay can be used to determine oxygen consumption rates of the 3 species of gastropod studied. Oxygen consumption rates per individual were significantly correlated with ETS$_i$. Therefore, in situ oxygen consumption rates for these species can be determined by taking individuals from a natural population, doing the ETS assay on an
Fig. 2. Predictive linear regressions of oxygen consumption per individual for 3 species of Lake Michigan gastropods. Filled circles represent mean (±1 SE) oxygen consumption rates of individuals. For *E. lizescens*, open circles represent results of in situ oxygen consumption measurements.

Table 2. Results of least squares linear analysis between the dependent variable (oxygen consumption per individual [mg O₂ ind⁻¹ h⁻¹]) and independent variables (experimental temperature [°C] and wet body mass [mg] of the snails tested).

<table>
<thead>
<tr>
<th>Species</th>
<th>Exp. temperature</th>
<th>Wet body mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>r²</em></td>
<td><em>p</em></td>
</tr>
<tr>
<td><em>Elimia lizescens</em></td>
<td>0.513 &lt;0.001</td>
<td>0.667 &lt;0.001</td>
</tr>
<tr>
<td><em>Physella integra</em></td>
<td>0.461 0.001</td>
<td>0.236 0.035</td>
</tr>
<tr>
<td><em>Stagnicola woodruffii</em></td>
<td>0.511 &lt;0.001</td>
<td>0.388 0.001</td>
</tr>
</tbody>
</table>

Individual, and using the calibration curves in Table 1. Although mass specific regressions may also be used to predict oxygen consumption rates in these snails (Table 1), per-individual ETS, is a better predictor.

Body mass and temperature were also correlated with individual oxygen consumption. This result was expected because many studies have used allometric parameters to predict oxygen consumption rates of gastropods (Åkerlund 1969, Fitch 1975) and other molluscs (Newell and Roy 1973, Bayne and Newell 1983). However, the results of the ETS assay show oxygen consumption rates are more strongly correlated with ETS, than with either wet-body mass or
temperature (Table 2). These results suggest that ETS can account for environmental factors such as temperature that may confound oxygen consumption measurement in the field.

The strength of the ETS–oxygen consumption relationship varied among species. Both *E. livescens* and *S. woodruffi* showed high correlation coefficients ($r^2 > 0.7$). However, *P. integra* had a lower correlation ($r^2 < 0.6$) value. This difference shows that the relationship between metabolic oxygen consumption and ETS activity is species specific (delGiorgio 1992). Therefore, the ETS assay must be calibrated to oxygen consumption for each species.

We used regression analysis to determine if the range of the R/E ratio remained constant over all the physiological and environmental parameters of the experiments. Considerable variance would be added to the predictive regressions if the R/E ratio showed dependence on either wet body mass or temperature. Also, dependence on these factors would suggest there was a separate relationship between oxygen consumption and ETS, for each size class or temperature. Our analysis showed no dependence between oxygen consumption and ETS, and either temperature (Fig. 3) or wet body mass (Fig. 4). Therefore, the ETS assay does not need to be calibrated to a specific size class or temperature, and the predictive regressions for each species are applicable over the range of conditions used in these experiments.

Predictive models should be tested statistically before use (Wahl and Stein 1991). Therefore, we tested ETS, as a predictor of individual oxygen consumption rates. Several independent tests of the regressions for each species confirmed that there was no significant difference between the line generated by the independent points and the 1:1 line. For all the independent tests the range in measured oxygen consumption rates was narrow compared to the predictive regressions. These data indicate the predictive linear regressions for *P. integra* and *S. woodruffi* are useful for estimating changes in oxygen consumption rates even in situations where large differences in oxygen consumption rates
do not exist. For *E. livescens*, however, the regression between measured and predicted oxygen consumption was non-significant. This result may have been caused by 1) small sample size and/or 2) narrow range in measured oxygen consumption of the independent test individuals (i.e., <0.5 the range of measured oxygen consumption of the predictive linear regression). The non-significant regression indicates the calibration line is not sensitive to small changes in oxygen consumption for this species. The range of in situ oxygen consumption did not exceed the limits of the predictive regression for field-collected *E. livescens*. Thus, the range of oxygen consumption and ETS activity from the laboratory experiments may encompass the typical natural range.

We varied temperature and body mass to obtain a range of oxygen consumption rates. The tight correlation between ETS and oxygen consumption rate allows us to use ETS to estimate oxygen consumption by snails even in conditions where direct measurement is difficult or impossible. Using the ETS assay, snails can be collected from the field and average oxygen consumption rates of the previous 7 d can be evaluated. The effects on oxygen consumption rates of pollution and fouling, and the effects of behaviors like migration, predator avoidance, and feeding can be evaluated. Using the ETS assay avoids the stress of collection and container effects. The ETS assay therefore provides us with a potentially valuable tool for studying oxygen consumption of organisms in a variety of situations.

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