Abstract

Shimps, Elizabeth Larissa. Hypoxia tolerance in two juvenile estuary-dependent fishes. (Under the direction of James A. Rice)

Hypoxia events, or low dissolved oxygen (DO) conditions, occur frequently in North Carolina estuaries during the summer. These events may have harmful effects on important fish stocks, including spot (*Leiostomus xanthurus*) and Atlantic menhaden (*Brevoortia tyrannus*), but their consequences are not well understood. As part of a larger study examining effects of hypoxia on juvenile estuary-dependent fishes, I investigated direct mortality due to hypoxia in juvenile spot and Atlantic menhaden. The objectives of these experiments were to determine how the extent of mortality varies with the severity of hypoxia and the duration of exposure, and to explore how vulnerability to hypoxia changes across species, temperature, and fish size.

Atlantic menhaden and spot were tested at two temperatures, 25° and 30°C, and three dissolved oxygen concentrations, 0.6, 0.9, and 1.2 ppm. Survival analyses were performed on the data relating survival rate of each species to dissolved oxygen concentration, duration of exposure, temperature, and fish size. The data were also analyzed using an LC_{50} approach for comparative purposes, and 12-hour LC_{50} estimates (concentrations causing 50% mortality) ranged from 0.9-1.1 ppm O_2. Spot and menhaden exposed to 1.2 ppm O_2 showed no mortality in 24 hrs at 25°C, and only 30-40% mortality at 30°C. In contrast, both species experienced 100% mortality in 2-6 hrs at 0.6 ppm O_2. There was a modest effect of size on hypoxia tolerance, with small spot being less tolerant than large spot, while the converse size effect was observed for menhaden. Spot were consistently less tolerant to hypoxia than menhaden and both species were less tolerant to hypoxia at 30°C than at 25°C. Preliminary

experiments showed that a 24-hour acclimation to sublethal levels of hypoxia caused significantly reduced mortality upon subsequent exposure to lethal hypoxia concentrations.

This study is part of a larger effort integrating lab experiments and field observations in a spatially-explicit, individual-based model to quantify changes in fish survival, growth and distribution in response to water quality changes. Results from this study indicate that while direct mortality due to hypoxia will vary with species, size, and temperature, mortality will likely only be substantial when these species are exposed to oxygen concentrations less than about 1 ppm O_2. Given the severity of hypoxia necessary to cause mortality and the ability of fish to behaviorally avoid hypoxia, direct mortality due to hypoxia may not occur on a large scale. Therefore, the greatest impacts due to hypoxia may be indirect, due to density-dependent effects on growth and survival as fish avoid hypoxic areas, or via mechanisms caused by stress imposed by sublethal hypoxic conditions alone or in concert with other stressors.

HYPOXIA TOLERANCE IN TWO JUVENILE ESTUARY-DEPENDENT FISHES

By

ELIZABETH LARISSA SHIMPS

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APPROVED BY:

_____
Dr. James A. Rice
Chair of Advisory Committee

_____ _____
Dr. Peter Rand Dr. Jason Osborne

Biography

Elizabeth Shimps was born and raised in Lewisville, North Carolina. She attended North Carolina State University for her undergraduate education and completed a B.S in Zoology and a B.S. in Natural Resources with a Marine and Coastal Resources Concentration in June 2001. In the fall of 2001, she continued with her education entering the Zoology graduate program at NC State to work under the direction of Dr. James A. Rice. Her coursework was completed at NC State, and her thesis research was performed at the NOAA Laboratory in Beaufort, NC.

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Introduction

Habitat loss is one of the primary threats to the sustainability of the nation's fisheries. Hypoxia contributes to habitat loss for fisheries resources by altering direct mortality and migration, reducing suitable habitats, changing food resources, increasing susceptibility to predation, and disrupting life cycles (Atwood *et al.* 1994). The United States Congress enacted the Sustainable Fisheries Act in 1996, which in 1998 required identification and description of essential fish habitat (EFH) in fishery management plans and minimization of deleterious effects on EFH (Schmitten 1999). EFH is defined as "those waters and substrate necessary to fish for spawning, breeding, feeding or growth to maturity" (USDOC 1996).

Identifying EFH and linking those habitat requirements to fishery production is difficult (Able 1999, Minello 1999). Many factors complicate our ability to quantify the direct and indirect effects of abiotic conditions like dissolved oxygen (DO) concentration on fish populations. These include difficulties in detecting effects of individual stressors on fish populations, nonintuitive population responses due to complex habitat, community interactions, and sublethal and cumulative effects on population responses (Rose 2000). For example, the highest survival of larval naked goby (*Gobiosoma bosci*) occurs at intermediate DO concentrations, rather than the highest concentrations (Rose 2000).

North Carolina has an extensive estuarine system that is susceptible to habitat loss due to hypoxia. North Carolina is experiencing major coastal growth; the human population in the eight counties bordering the Atlantic Ocean increased 26% from 1990 to 2000 (US Census Bureau 2003). In addition, the 28 counties surrounding the Albemarle-Pamlico Estuarine System contain 48% of the state's cropland and there has been a significant increase in livestock production in this area (Copeland and Grey 1989). These factors,

combined with disruptive events like hurricanes, contribute heavily to both point and non-point source nutrient loading to North Carolina's coastal waters and are often factors in large-scale anoxia and fish kill events (Mallin *et al.* 2000).

Many of North Carolina's estuaries, including the Neuse, New, and Pamlico, are considered eutrophic and exhibit symptoms such as phytoplankton blooms, bottom-water hypoxia and anoxia, and fish kills. Hypoxia causes large portions of the Neuse River estuary to be unsuitable habitat for most fish at various times in the summer, possibly resulting in higher densities and increased competition in normoxic areas (Eby and Crowder 2002).

Eutrophication mitigation efforts in North Carolina have included a 1988 statewide ban on phosphorous-containing detergents and installation in 1992 of a waste treatment system in the world's largest phosphate mine reducing effluent phosphate concentrations by 90% (Mallin *et al.* 2000). In 1997 the state legislature mandated a 30% reduction in nitrogen load in the Neuse River Estuary (Borsuk *et al.* 2001). By decreasing anthropogenic nutrient loading, North Carolina anticipates ameliorating the severity of hypoxia in its estuaries.

Anthropogenic nutrient loading from point and non-point sources stimulates algal production, creating biological oxygen demand that can result in hypoxia. Hypoxia events can be triggered by nutrient-enhanced phytoplankton blooms in a matter of weeks, in days by increased organic matter input from flushing after storm events (Paerl *et al.* 1998), or in only hours when estuaries experience calm conditions combined with biological oxygen demand. Many physical factors also contribute to hypoxia's occurrence. Increased salinity and temperature reduce the oxygen-saturation concentration, making hypoxia events especially likely in summer. Warm temperatures and stratification are conducive to creating bottom-water hypoxia events, and often occur in concert with high freshwater discharge and low

wind stress, strengthening vertical stratification. The duration and expanse of hypoxia events is largely determined by the balance between oxygen uptake and frequency of strong wind events. Estuaries most commonly experience bottom water hypoxia in deeper areas, but hypoxia intrusion into nearshore shallow waters occurs periodically due to winds and lateral water movement causing upwelling of hypoxic bottom waters.

Fish can become trapped in hypoxic waters if conditions change rapidly or there is no escape route (Breitburg *et al.* 1997, Paerl *et al.* 1999). In the Chesapeake Bay, DO concentrations can change rapidly, with readings sometimes varying by 0.5 ppm or more over 15 min (Breitburg 1992). Field studies indicate an absence of most fish species in hypoxic waters if an escape route is available (Schwartz *et al.* 1981, Pihl *et al.* 1991, Eby and Crowder 2002) and laboratory experiments have shown that many fish species actively avoid hypoxia (Wannamaker and Rice 2000).

Juvenile spot and Atlantic menhaden were selected as the experimental organisms for this study because they are ubiquitous in estuaries during the summer when hypoxia occurs. The two species are also commercially important fish in North Carolina, with landings of over $5 million in 1998 (NC DMF 1999), and are important members of the estuarine food web. Spot and Atlantic menhaden larvae enter estuaries in the winter and spring and spend the juvenile phase of their lives in the estuaries before leaving in late summer or fall. Spot and Atlantic menhaden differ in their usage of the estuaries; juvenile spot are benthic, grazing generalists and juvenile Atlantic menhaden are pelagic, schooling, filter feeders. Juvenile Atlantic menhaden form dense spatiotemporally dynamic nursery aggregations in estuaries during the summer that are correlated to phytoplankton biomass, a distribution pattern attributed to optimizing feeding and growth (Friedland *et al.* 1996). Because Atlantic

menhaden are a pelagic species, Burkholder *et al.* (1999) suggested that they may encounter reduced usable habitat during bottom-water hypoxia events but that mortality events are most likely due to multiple stressors and not solely due to hypoxia. Paerl *et al.* (1999) contend that menhaden are likely to be trapped in hypoxic waters because of their tendency to aggregate in phytoplankton rich areas, the same areas most likely to experience hypoxia events.

The effects of estuarine hypoxia are especially important to consider as estuaries provide nursery habitats for many economically and ecologically important species. The primary objective of this research is to consider the mortality effects due to hypoxia on two juvenile estuary-dependent species, determining how the extent of mortality varies with the severity of hypoxia and the duration of exposure, and exploring how vulnerability to hypoxia changes across species, temperature, and fish size. First, I hypothesized that Atlantic menhaden may be less tolerant to hypoxia than spot as menhaden predominate fish kills presumed to be attributable to hypoxia in North Carolina (NC DWQ 2003). Second, higher temperatures may cause a more severe mortality response due to hypoxia because of increased metabolic rates and oxygen requirements. Third, mortality due to hypoxia may increase as fish size increases due to greater oxygen usage. Some work has previously been done on hypoxia tolerance of spot and Atlantic menhaden (Table 1); however, none of this work comprehensively describes hypoxia tolerance, relating mortality to hypoxia severity, exposure duration, temperature, and fish size.

A second objective of this research was to use survival analyses, or time-to-effect methods, for primary data analysis to demonstrate the many advantages survival analyses offer compared to more commonly used concentration-effect methods. Burton *et al.* (1980) performed the most comprehensive dissolved oxygen mortality studies for spot and Atlantic

menhaden (Table 1). However, those studies examined mortality in terms of LC_{50} estimates (concentrations causing 50% mortality), a common means of expressing mortality data, but the standards generated are based on DO concentrations that are lethal to fish, and are thus of questionable safety in protecting fish populations (Seager *et al.* 2000). Despite the risks inherent in using concentration-effect methods like the LC_{50}, these methods are the predominant form of analysis in aquatic toxicology.

LC_{50} estimates are statistically reliable, with the narrowest 95% confidence interval along the dose-response curve (Newman and Dixon 1996). Therefore they generate uniform and well-defined statistical information on which to base regulatory decisions (Newman and Aplin 1992). However, these methods are so ingrained into the regulatory framework that the merits of other approaches are often not critically considered (Newman and Dixon 1996).

Concentration-effect methods have many limitations that can be overcome by using other analytical approaches. Confidence in concentration-effect estimates decreases at percent mortalities away from 50%, so concentration-effect methods may no longer be the most statistically reliable and effective model choice for evaluating more ecologically relevant levels of a substance that cause less than 50% mortality (Newman and Dixon 1996). Another potential problem with using concentration-effect methods is the lack of information about covariates (e.g., mass, temperature), as they are seldom incorporated and are usually intentionally minimized. These actions enhance the precision of an LC_{50} estimate but limit predictive ability about the effects to individuals in the field, as these individuals do exhibit and experience natural variation (Newman and Aplin 1992). LC_{50} estimates also have a limited ability to predict toxicity over time as an LC_{50} must be estimated at a series of times

to gain information about multiple exposure durations, whereas observing the pattern of mortality over time could increase statistical power (Newman and Aplin 1992).

Survival analyses, or time-to-effect methods, offer many advantages over the more commonly used concentration-effect methods. Survival analysis increases statistical power because more data are collected, with time-to-effect (i.e., time to death) of every individual being noted (Newman and Aplin 1992). The focus of survival analysis on time response rather than dose response increases the precision of estimates at mortality percentages of less than 50% (Newman and Dixon 1996) and allows for description of survivorship patterns at all exposure times instead of only one exposure time (Dixon and Newman 1991). Covariates are more easily included in survival analysis due to its enhanced statistical power compared to concentration-effect methods (Newman and Dixon 1996). Inclusion of covariates allows survival analysis to examine the potential effects of different environmental conditions or organism characteristics on acute toxicity (Dixon and Newman 1991), and thus provides more ecologically meaningful estimates of lethal effect than concentration-effect methods (Newman and Dixon 1996). All of these factors contribute to improving the ability to predict field effects on fish populations from lab data (Newman and McCloskey 1996).

Support for the use of a survival analysis approach is bolstered by its extensive use in other fields including epidemiology, clinical medicine, and engineering (Dixon and Newman 1991). In addition, survival analysis can easily be included in a study with the ultimate goal of describing an LC_{50} by simply making more frequent observations of mortality. Thus, survival analysis does not detract from LC_{50} estimation, but allows for greater data analysis possibilities while still producing an LC_{50} that is easily comparable with the existing literature (Sprague 1969, Newman and Aplin 1992). Furthermore, survival analysis can be

implemented using common statistical software packages including SAS and S-plus (Dixon and Newman 1991).

Methods

Species Collection and Maintenance

Both species were collected from inshore estuarine areas around Beaufort, North Carolina. Juvenile Atlantic menhaden were collected using a cast net, and juvenile spot were collected using a beam trawl or otter trawl. Both species were held in outdoor tanks, with Atlantic menhaden in circular tanks, at 15 ppt salinity and approximately a 14-hour light:10-hour dark photoperiod at normoxia (~7 ppm O_2). Temperatures in the outdoor tanks ranged from 23-29°C. Atlantic menhaden were fed *ad libitum* with finely ground commercial fish food and spot were fed *ad libitum* with pelleted commercial fish food.

Due to the limited availability of wild spot I used fish hatched and reared in the lab to supply 1/3 of the spot for all of the trials at 30°C and for the 25°C trial at 1.2 ppm O_2. Hatchery spot were reared at the NOAA Laboratory in Beaufort, NC and held in an indoor tank at the same conditions as the wild fish. The average size of the hatchery spot was 47.8 mm standard length (SL) (± 6.87 mm SD), while the average size of the wild spot was 65.9 mm SL (± 9.47 mm SD).

Mortality Trials

Lethal hypoxia tolerance of spot and Atlantic menhaden was determined through a probability of mortality study conducted at 15 ppt salinity and at two temperatures, 25 and 30°C, representing typical summertime estuary conditions. Each species underwent trials at

both temperatures and at three different levels of hypoxia, 0.6, 0.9, and 1.2 ppm O_2. Each trial used about 150 fish per treatment, a sample size determined by modeling confidence in survival time estimates at different DO concentrations and sample sizes using preliminary data from trials with pinfish (*Lagodon rhomboides*) (B. Miller and J. Rice, unpublished data) using Analytica for Windows Version 2.0. Thirty additional fish were used as controls for each treatment.

An additional trial was run to test the effects of acclimation to hypoxia on mortality response. In this trial I tested the tolerance of both Atlantic menhaden and spot to 0.6 ppm O_2 at 25°C, following a 24-hour acclimation to 1.2 ppm O_2.

Fish were acclimated outdoors to most experimental conditions, including the photoperiod and salinity, for a minimum of one week. Fish were then acclimated to the experimental setup, 12 62-L recirculating tanks, at normoxia to minimize handling effects and to acclimate to the experimental temperature. About 30 fish were placed in each tank, allowing for two treatments to be tested at one time. For 25°C experiments there was a 24-hour acclimation period, and for 30°C trials there was a 48-hour acclimation period. Fish were deprived of food beginning 24 hours prior to the experimental period to avoid effects of digestion on the metabolism of the fish (Durbin *et al.* 1981).

After acclimation, 10 of the 12 tanks began the DO treatments, with the remaining two normoxic tanks serving as the control for non-hypoxia-related mortality. Tanks stopped recirculating and were static for the duration of the experiment. DO levels were reached by bubbling N_2 directly into the tanks, a process taking about 1 hour. I considered this an appropriate rate because previous research showed that the rate of DO reduction Atlantic menhaden are exposed to does not affect the absolute DO concentration causing death

(Burton *et al*. 1980). DO levels were maintained by bubbling N_2 or air into the tanks as necessary. The tank surfaces were covered with lids made of Styrofoam and plastic sheeting to minimize oxygen diffusion. DO measurements were taken in different areas of the tanks prior to the experiments to test for variability within the tank and DO concentrations were found to be consistent throughout. During each trial, DO levels were monitored with a YSI Model 52 DO meter probe every 15 minutes for the first 6 hours and every 30 minutes thereafter. Probe measurements were calibrated using the air calibration method. Using the calculations provided in the YSI Model 52 Dissolved Oxygen Meter Operations Manual, I estimated measurement error associated with the instrument components, probe accuracy, and calibration of the DO probe to be ± 0.01 ppm O_2 at 25°C and ± 0.03 ppm O_2 at 30°C.

The tanks were continuously monitored, and the time of death was recorded for each fish. Upon death, each fish was removed from the tank, weighed and measured, so that size could be used as a covariate in the analysis. The trials were terminated after 24 hours, and any remaining live fish were weighed and measured. A 24-hour experimental period was warranted as Sprague's (1969) collection of LC_{50} estimates shows that lethal thresholds for static tests are often evident within one day, although he also recommends that tests should continue until the shape of the toxicity curve is well established (which occurred within 24 hours in this study).

There were a few exceptions to the 24-hour experimental period. The spot and Atlantic menhaden experiments at 25°C and 0.9 ppm O_2 were ended after 12 hours due to widely fluctuating DO concentrations after that point. Also, three tanks in the Atlantic menhaden experiment at 30°C and 1.2 ppm O_2 were ended at 17 hours and 40 minutes because DO concentrations in these tanks dropped severely.

Data Analysis

SAS Version 8.2 was used for all data analysis (example analyses shown in Appendix). Only fish exposed to hypoxic conditions were used in the data analysis. Any trial fish remaining alive after the experimental period were right censored, meaning only data on minimum survival time were analyzed. Fish from different tanks were pooled for treatment analysis as separate tanks were used solely to facilitate data collection (i.e., tanks were not replicates) and Kaplan-Meier analysis found no consistent tank effect among the experiments.

Data were modeled using the Cox regression method, a semiparametric proportional hazards model. This model was chosen instead of an accelerated failure time model because it still assumes a parametric form for the explanatory variables but, unlike the accelerated failure time model, it allows the survivor function to have an unspecified form.

In the Cox regression model each individual's survival time is assumed to have its own hazard function

$$h_i(t) = h(t;z_i) = h_o(t) \bullet \exp(z'_i\beta)$$

where $h_o(t)$ is an arbitrary and unspecified baseline hazard function, z_i is the vector of measured explanatory variables for the *i*th individual (e.g. DO, temperature, size), and β is the vector of unknown regression parameters associated with the explanatory variables, which is assumed to be the same for all individuals (SAS OnlineDoc V8 1999). The partial likelihood function (Cox 1975, Cox 1972) estimates β, eliminating the unknown baseline hazard $h_o(t)$ and accounting for censored survival times.

The survivor function is

$$S(t; z_i) = [S_0(t)]^{\exp(z'_i \beta)}$$

and

$$S_o(t) = \exp(-\int_o^t h_o(u)du)$$

is the baseline survivor function (SAS OnlineDoc V8 1999). The survivor function is essentially the probability of surviving beyond t and at t = 0 the survivor function must equal one. My experiments violated this assumption because some fish died as the DO concentration was being reduced but before the target DO was reached (i.e., survivorship was < 1 at t = 0); however, I adjusted for this occurrence by assigning fish that died before the experiment started a time-to-death equal to 0.00001 minutes. This allowed the Cox regression model to fit my data and should not affect any biological interpretations as these fish were the first to die in the experiments and this is accounted for in the model.

First, I tested the assumption that hazards are proportional between species by plotting the log (-log) survivor functions for each species. The functions were not parallel, indicating that hazards are not proportional between species. Therefore, separate models were generated for Atlantic menhaden and spot and the following explanatory variables were included in each model: actual mean DO concentration each fish experienced until its death or censoring, fish mass, and water temperature.

Tied data (i.e., multiple fish dying at the same time) were handled by assuming tied event times occurred before censored times of the same value or larger values and then calculating the exact conditional probability (SAS OnlineDoc V8 1999). This method was

used because there were a large number of tied data, and the default method, Breslow's approximation, can be very poor when the data are heavily tied (Allison 1995).

Akaike's information criterion (AIC) was used to select the models with the best fit, or smallest AIC value. The simplest Cox regression model included the direct effects of DO, temperature, and mass, and indicated each of these variables had a significant effect on survival. Other possible models were run testing interactions and polynomial terms, and the fit of those models was compared using AIC, residuals, and comparison to the raw data. Ultimately, the Atlantic menhaden and spot models that were chosen included only the three main effects. While the AIC values for these models, 5301 for Atlantic menhaden and 5536 for spot, were slightly higher than some more complex models, their simplicity and the ability to use comparable models for both species was beneficial. The log (-log) survival distribution function was plotted against the log of Cox-Snell residuals as an additional check on model fit (Collett 1994).

Cumulative hazard functions were plotted for each species' model to see how the hazard changes over time. The -log of survivor function estimates (SFE) corresponding to the means of the explanatory variables were plotted against time. For spot the mean explanatory variables were a 5.82g fish exposed to 0.93 ppm O_2 at 27.5°C, and for Atlantic menhaden they were a 5.99g fish exposed to 0.91 ppm O_2 at 27.6°C.

LC_{50} values of lethal hypoxic levels were calculated for each species-temperature combination tested at 12 and 24 hours using probit analysis (Newman 1995). DO concentrations were $\log_{10}$ transformed for this analysis. An empirical transformation of the data was necessary to avoid infinite parameter estimates in the probit analysis, so ½ success and ½ failure were added to each data point (McCullagh and Nelder 1989). The convergence

of the algorithm for the 12-hour spot dataset at 25°C was questionable, so the LC_{50} estimate from this analysis was not reported. Treatment differences were also investigated using probit analysis.

The mortality response to hypoxia was estimated using the nonparametric Kaplan-Meier method of estimating survivor functions for all experiments including the acclimation experiment. For all trials that do not contain censoring or are only censored at one time after all observed event times (i.e., at the end of the 24-hour trial), the Kaplan-Meier estimator equals the proportion of observations with event times > t. However, when censored times are less than time to death for some fish in the same trial, those censored individuals could have died before later event times, and the proportion of observations with event times > t could be biased downward without using the Kaplan-Meier estimator to take this into account (Allison 1995). This procedure generated estimated survival probabilities and 95% confidence intervals for each treatment tested.

Results

Average DO concentrations were within 0.04 ppm of the target concentrations in each treatment, and were held to an acceptable level of variability within each treatment (Table 2), with 90% of DO measurements being within 0.2 ppm of the target concentration. The control fish, held at normoxia but exposed to all other treatment conditions, experienced only 0.67% mortality (2 fish died), indicating that mortality observed in the hypoxic treatments was due to the low DO concentration and not other stressors.

LC_{50} Analysis

While most treatments ran the entire 24-hour experimental period, three required that data be censored earlier, so LC_{50} analysis was performed at 12 hours as well as 24 hours for each experiment to obtain estimates from a time encompassed by all experiments. The 12-hour LC_{50} estimate for spot at 25°C could not be determined accurately due to the nature of the data (survival was 100% at the highest DO level and at or near 0% at the other two DO levels), so it was not included. Results were similar for both 12 and 24-hour analyses, consistent with the observation that the majority of mortality occurred during the first few hours of hypoxic exposure. Therefore, only 12-hour results are shown (Figure 1) because they represent the experimental period to which all organisms were exposed. Each treatment for which an LC_{50} was generated was significantly different ($P<0.05$) from the other treatments. Atlantic menhaden at 25°C exhibited a less severe mortality response across all DO concentrations tested than they did at 30°C and both those treatments showed a less severe response than spot at 30°C (Figure 1).

Survival Analysis

For both Atlantic menhaden and spot, hazard decreases over time (Figure 2), indicating that the mortality rate is higher near the beginning of the experiments than at the end. DO, temperature, and fish mass all had significant effects on the hazard function for both Atlantic menhaden and spot (Table 3). The nature of these effects is indicated by their hazard ratios; if the hazard ratio of a variable is >1, an increase in the variable increases the hazard rate, and if the hazard ratio is <1, an increase in the variable decreases the hazard rate (SAS OnlineDoc V8 1999). For both species hazard decreased with increasing DO, but

increased with increasing temperature. However, the effect of mass was different for the two species; hazard increased with increasing mass for Atlantic menhaden, but decreased with increasing mass for spot.

The hazard ratio for each covariate can be roughly interpreted as a measure of relative risk (Table 3). All of the covariates in the models for both species are quantitative (i.e., no categorical covariates), so the estimated percent change in the hazard for a 1-unit increase in a covariate can be obtained by subtracting 1 from the hazard ratio and multiplying by 100 (Allison 1995). Thus, for each gram an Atlantic menhaden increases in mass the mortality hazard increases by 8.3% (i.e., [1.083 – 1] • 100), whereas a similar increase in mass for spot results in a 4.5% decrease in the mortality hazard. With every 1°C increase in temperature, hazard increases 23.9% for Atlantic menhaden and 10.9% for spot. Of all three variables, DO has the greatest effect on mortality hazard; an increase in DO concentration of 0.1 ppm reduces hazard about 48% for both Atlantic menhaden and spot.

I used the Cox regression models to generate response surfaces showing how probability of survival varies as a function of DO concentration and exposure time for Atlantic menhaden and spot at 25 and 30°C (Figure 3). Mass was held constant for each surface by using the mean mass for each species (Table 4). The same general trend is evident across all the response surfaces; probability of survival decreases sharply over the first few hours and flattens out towards the end of the experimental period, with survival ranging from 0 to 100% over a very narrow DO range from about 0.5 – 1.5 ppm. Generally, at any given combination of time and DO concentration, probability of survival is lower at 30°C than at 25°C and higher for Atlantic menhaden than for spot.

I selected the most appropriate models using AIC as a measure of model fit. The residual plot of the log (-log) survival distribution function against the log of Cox-Snell residuals provided an additional check on model fit, and both species' models produced an approximately straight line consistent with good model fit (Collett 1994). Finally, the chosen models were visually compared to Kaplan-Meier survivor functions to assess the fit of the Cox regression model's predicted probabilities of survival to the Kaplan-Meier estimates (Figure 4). Again, mean mass for each species at each temperature were used. The Cox regression model appears to be an unbiased estimator of survival probability and fits fairly well for Atlantic menhaden at 25 and 30°C and for spot at 30°C, but not as well for spot at 25°C.

Because the two species were modeled separately, there is no hazard ratio to indicate the species effect on survival. Therefore, I graphed Atlantic menhaden and spot survival probabilities from each model together to visually compare them at 25 and 30°C. At every temperature and DO concentration, Atlantic menhaden showed either a higher percent survival after 24 hours or a longer time to 100% mortality than spot (Figure 5). At 25°C and 0.6 ppm O_2, survival was predicted to drop to below 3% after 2 hours for spot and after 5 hours for Atlantic menhaden. At 25°C and 0.9 ppm O_2, predicted Atlantic menhaden survival was 51% after 24 hours, while predicted spot survival was only 12%, and after 24 hours at 1.2 ppm O_2, predicted survival was 91% for Atlantic menhaden and 74% for spot. A similar pattern is evident at 30°C, although the effect is much smaller at 0.9 ppm O_2, as after 24 hours predicted survival was 14% for Atlantic menhaden and 3% for spot. These data indicate that spot consistently exhibited a more severe mortality response to hypoxia than did Atlantic menhaden.

To better evaluate the effect of temperature on survival of each species I graphed predicted survival probabilities at 25 and 30°C for the mean size of each species at each experimental DO level (Figure 6). At every DO level, survival was higher at 25°C than at 30°C for both species. After 24 hours at 25°C and 0.9 ppm O_2, absolute predicted survival for Atlantic menhaden was 37% greater than at 30°C. The difference was less pronounced at both higher and lower DO levels. At 1.2 ppm O_2, absolute survival was15% greater at 25°C than at 30°C and at 0.6 ppm O_2 there was still 0.008% predicted survival after 24 hours at 25°C while survival was predicted to decline to 0 after 14 hours at 30°C. For spot, the temperature effect was more consistent across DO levels. After 24 hours at 25°C and 0.9 ppm O_2, absolute predicted survival was 9% greater than at 30°C, while at 1.2 ppm O_2 absolute survival was13% greater at 25°C than at 30°C. At 0.6 ppm O_2 and 25°C, predicted survival declined to 0 after 13 hours, while at 30°C survival was predicted to decline to 0 after 3 hours. Both Atlantic menhaden and spot exhibited a more severe mortality response due to hypoxia at 30°C than at 25°C, although this difference was slightly more pronounced for Atlantic menhaden.

Similarly, I examined mass effects by graphing predicted survival probabilities for each species at 25 and 30°C with DO concentration held constant at the mid-range treatment level of 0.9 ppm (Figure 7). Smaller Atlantic menhaden were consistently more tolerant to hypoxia than larger fish, although this effect was small with predicted survivals being 8-9% greater for fish at the 10^{th} mass percentile than fish at the 90^{th} mass percentile at both 25 and 30°C. Spot exhibited the converse effect; smaller fish were less tolerant to hypoxia than larger fish, although this effect was also small with predicted survivals being 2-5% less for fish at the 10^{th} mass percentile than fish at the 90^{th} mass percentile at both 25 and 30°C.

While the absolute effect of mass on survival was constant throughout the experiments, the relative effect of mass on predicted survival grew quite large when total survival was low.

Effects of Acclimation

A 24-hour acclimation to sublethal hypoxic levels of 1.2 ppm O_2 significantly increased survival time of Atlantic menhaden and spot at 25°C when they were subsequently exposed to 0.6 ppm O_2 (Log-rank statistic: $P < 0.0001$). While the acclimation experiment ran for only 3 hours due to logistical constraints, this time period appeared to be sufficient as it encompassed substantial effects. After 3 hours, cumulative mortality for acclimated spot and menhaden was 7-20%, while cumulative mortality for spot and menhaden that went from normoxia directly to 0.6 ppm O_2 within 1 hour was 90-100% (Figure 8).

Discussion

Analysis Approach

Experiments were conducted in a manner that allowed both LC_{50} and Cox regression survival analyses to be done, thus capturing the benefits of both approaches. There are many advantages to including survival analysis. Survival analysis can generate better survivorship estimates than the LC_{50} approach near complete mortality and complete survival (Newman and Dixon 1996), as illustrated by the 12-hour 5% mortality estimates obtained from both models for these data. The LC_{05} estimates and survival analysis estimates differed by as much as 0.20 ppm O_2, and the confidence intervals widened from a maximum of 0.06 ppm O_2 for LC_{50} estimates to a maximum of 0.19 ppm O_2 for LC_{05} estimates, indicating that the suitability of the LC_{50} approach for making survivorship predictions decreases as mortality

diverges from 50%. Also, survival analysis does not substantially detract from estimates of 50% mortality, generating similar estimates as the LC_{50} approach; the 12-hour estimates for the LC_{50} and for 50% mortality using survival analysis differed by no more than 0.04 ppm O_2, indicating that either model can approximate the concentration at which 50% mortality will have occurred over a set period of time.

A survival analysis approach may also make an analysis possible when the mortality agent causes such an acute response that it is difficult to derive precise LC_{50} estimates due to the narrow range of effect that is difficult to subdivide. My experiments exemplify the problems encountered with an acute toxicant as the entire range of effect was encompassed within a range of about 1 ppm O_2. A precise LC_{50} estimate would require at least five different tested concentrations within this range and I only used three concentrations. The variability in my DO measurements suggests that while the three concentrations tested were sufficiently separated (Table 2), further subdivision within this range of DO concentrations would probably not have yielded distinctly different DO treatments. I used a simple, inexpensive method of controlling DO concentrations by bubbling N_2 into the tanks; a more complex, possibly computerized method would likely be necessary to refine the variability in DO concentrations adequately to allow for testing of more DO concentrations within this narrow range.

Another benefit of using survival analysis is that it allows for easy incorporation of covariates, such as the size covariate included in my analyses. Covariate incorporation can be limited in LC_{50} analysis because LC_{50} test protocols often attempt to minimize differences in test subjects to enhance the precision of the estimates, a technique that actually reduces the predictive power of estimates for field populations (Newman and Aplin 1992). Also, to test a

size effect using an LC_{50}, size groupings of interest would have to be determined, and more experiments would have to be performed because an LC_{50} for each size class of interest would be needed and the effect would be estimated by comparing those estimates. Survival analysis allows for covariate analysis within an experiment, which is preferable because the number of trials and number of organisms required to generate the information of interest is minimized and size can more appropriately be treated as a continuous variable, rather than as arbitrary discrete groupings.

Survival analysis also provides more ecologically relevant information by generating continuous estimates of mortality over the full range of the treatment variable. Survival analysis generates survivor and hazard functions which describe survivorship at all times during the experiment, unlike LC_{50} analysis which only generates survivorship information about discrete, predefined exposure times. Furthermore, experiments conducted according to the traditional LC_{50} protocol can be evaluated using survival analysis by simply noting time-to-death of each individual during testing, so survival analysis need not detract from the LC_{50} measurements of interest and can provide enhanced data interpretation. Therefore, it is advantageous to generate survivor and hazard functions when analyzing mortality data, as the scope of an experiment can be expanded from one dealing with toxicological risk to one also addressing ecological risk.

Hypoxia Tolerance

These experiments investigated the mortality effects due to hypoxia on two juvenile estuary-dependent species, exploring how vulnerability to hypoxia changes across species, temperature, and fish size, and determining how the extent of mortality varies with the

severity of hypoxia and the duration of exposure. Atlantic menhaden were more tolerant to hypoxia than spot and both species were more tolerant to hypoxia at 25°C than at 30°C. Size also had an effect on hypoxia tolerance although it differed between species, with smaller Atlantic menhaden being more tolerant and smaller spot being less tolerant. A preliminary experiment suggested that acclimation to sublethal hypoxia levels increases hypoxia tolerance of both species. Regardless of covariate effects, mortality due to hypoxia occurred in a very narrow DO range and at very low DO levels.

Spot exhibited a more severe mortality response to hypoxia than Atlantic menhaden at 25 and 30°C (Figure 5), a finding that suggests benthic species may be less tolerant to hypoxia than pelagic species, which is contrary to some ideas found in the literature. Breitburg (1994) found that naked gobies, a benthic species, did not show escape responses until DO dropped below 0.75 ppm and adult male naked gobies experienced 100% survival when exposed to 0.7 ppm O_2 for 7 hours each of 7 days whereas adult bay anchovy (*Anchoa mitchilli*), a pelagic species, were less tolerant to low DO with an estimated 96-h LC_{50} value of 1.85 ppm O_2. However, given my results, information on more species will be needed to determine if benthic and pelagic species typically differ in their hypoxia tolerance.

Burton *et al*. (1980) found that spot were more tolerant to hypoxia than Atlantic menhaden, estimating the 24-hour LC_{50} of spot at 0.67 ppm O_2 and of Atlantic menhaden at 0.88 ppm O_2. The discrepancies between these findings and those from my own experiments may be attributable to different conditions and fish used in the two experiments. While both experiments used similar temperature, photoperiod, and spot size, I used a higher salinity (15 ppt compared to 6.9 ppt) and much smaller menhaden (mean ± 1 SD = 5.99 ± 2.70g compared to18.1 ± 3.9 g). Furthermore, although the size range of menhaden I used was

limited (Table 4), my data suggest that larger menhaden are more vulnerable to hypoxia, a trend that could partially explain these discrepancies.

Atlantic menhaden are generally thought to be very susceptible to hypoxia. This presumption is supported by the North Carolina Division of Water Quality fish kill database, which shows that Atlantic menhaden comprise a large percentage of estuarine fish kills presumed attributable to hypoxia. However, this is not true of all fish kill events, as during the summer of 2003 there were at least two large fish kills caused by hypoxia in the Neuse River that were predominantly spot (NC DWQ 2003). Atlantic menhaden may be particularly susceptible to hypoxia because they aggregate in phytoplankton rich areas, the areas most likely to experience hypoxia events (Paerl *et al.* 1999). However, the congregation of menhaden in areas likely to have low DO concentrations combined with their dense schooling behavior that is likely to further lower DO levels in the immediate area may have the opposite effect, increasing Atlantic menhaden's tolerance to hypoxia due to acclimation.

Both species displayed a more severe mortality response due to hypoxia at 30°C than at 25°C (Figure 6). The two temperatures tested, 25 and 30°C, are within the range of temperatures both Atlantic menhaden and spot are likely to experience during summer in North Carolina estuaries.

The literature shows either no effect of temperature on hypoxia tolerance of fishes or decreasing tolerance with increasing temperature. Southern flounder (*Paralichthys lethostigma*) did not exhibit differing sensitivity to DO concentration at 6.1°C, 14.4°C, or 25.3°C, with fish totally withdrawing from the hypoxic region to well-oxygenated waters at 0.68-1.09 ml/L O_2 (Deubler and Posner 1963). In lethal hypoxia experiments on Atlantic cod

(*Gadus morhua*), temperature did not affect hypoxia tolerance, possibly because the temperature range (2°C – 6°C) was not sufficient to show such effects (Plante *et al.* 1998).

Other experiments showed that increasing temperature decreased hypoxia tolerance, a finding consistent with my experiments. For Atlantic cod, 50% mortality occurred at 0.5 ppm O_2 at 5°C and at 2.3 ppm O_2 at 17°C (Schurmann and Steffensen 1992). Juvenile Atlantic sturgeon (*Acipenser oxyrinchus*) held at 3 ppm O_2 for three days experienced 22% mortality at 19°C and 92% mortality at 26°C (Secor and Gunderson 1998). It makes sense that hypoxia tolerance would decrease as temperature increases because higher temperatures increase the metabolic rates of fish, thus increasing oxygen requirements (Breitburg 2002).

Size had a significant effect on hypoxia tolerance, with Atlantic menhaden showing a decreasing tolerance to hypoxia as fish mass increased and spot exhibiting the opposite effect, showing an increasing tolerance to hypoxia as fish mass increased (Fig 7). The size effect was small in absolute terms for both species, on the order of a 2-9% difference in survival probabilities between the 10^{th} and 90^{th} mass percentiles. This could be partially attributable to the narrow size range encompassed by the fish tested (Table 4); size effects may be more pronounced outside these ranges. However, this small absolute difference translated into a large relative difference between survival of the 10^{th} and 90^{th} mass percentiles after 24 hours, with about a 50% difference in total survival for all experiments except Atlantic menhaden at 25°C.

Size effects on hypoxia tolerance in the literature are mixed. One idea is that larger fish typically use more oxygen per hour than smaller fish (Moyle and Cech 2000), so larger fish may be more susceptible to hypoxia. These observations are consistent with my findings for Atlantic menhaden, as larger fish were consistently less tolerant to hypoxia than smaller

fish. The greater susceptibility to hypoxia by large fish is supported by observations of large fish showing stress while smaller fish exhibit no response in hypoxic waters (Hunn and Schnick 1990) and juvenile brown shrimp (*Penaeus aztecus*) (1.37g) having significantly lower lethal DO levels than sub-adult brown shrimp (6.12g) (Kramer 1975).

However, I observed the converse effect for spot, where smaller fish were consistently less tolerant to hypoxia than larger fish. There are also instances of this effect reported in the literature. Shepard (1955) found that small brook trout (*Salvelinus fontinalis*) died more quickly than large brook trout. Hughes (1984) found that fish increase the difference between their standard metabolic level and their maximum activity level as body mass increases. In order for fish to have the ability to take in the oxygen needed to fuel these expenses, gill area might increase more rapidly than mass as is seen in salmon (Brett and Glass 1973). These findings suggest that larger fish might be able to acquire sufficient oxygen for survival at lower DO concentrations than smaller fish. Other studies have found no correlation between size and resistance to hypoxia in cutthroat trout (*Oncorhynchus clarki*) (Wagner *et al.* 2001), rainbow trout (*Oncorhynchus mykiss*), or perch (*Perca fluviatilis*) (Alabaster *et al*. 1957). The variation in reported findings in the literature and the differences I found in size effects for spot and Atlantic menhaden suggest that fish size can have an effect on hypoxia tolerance, but that the effect may be species-specific.

Fish size could still impact mortality indirectly in species for which size does not appear to have a direct effect on hypoxia tolerance. Fish swimming speeds typically scale with body size so size may affect a fish's ability to escape hypoxic areas. For example, in the Chesapeake Bay many large juvenile and adult naked gobies survived hypoxic intrusions by

temporarily migrating inshore while these sudden hypoxic events caused almost complete mortality in younger juveniles (Breitburg 1992).

Fish acclimated for 24 hours to sublethal hypoxic levels before being exposed to 0.6 ppm O_2 exhibited 80% greater proportion survival compared to fish experiencing DO dropping from normoxia to 0.6 ppm O_2 in 1 hour (Figure 8). The majority of my experiments did not test for acclimation effects; however, during my 24-hour trials over 90% of the mortality occurred within the first 5 hours of the experiment. A similar trend was evident in Magaud *et al.*'s (1997) 24-hour mortality trials exposing juvenile rainbow trout to varying levels of hypoxia. Fish either died within the first few hours of treatment or did not die during the experimental period, which the authors suggested might be due to acclimation of the trout to hypoxia.

The ability of fish to acclimate to hypoxia and the period of time over which an acclimation effect is evident are matters of debate in the literature. There is some evidence indicating that fish can acclimate to hypoxia. Davis (1975) stated that there were many indications of fish acclimating somewhat to decreased DO levels. Shephard (1955) found that acclimation increased resistance times in fish exposed to lethal DO levels five-fold. In three species of freshwater fish, resistance to hypoxia increased when fish were acclimated to low DO levels over 11 to 15 days as compared to oxygen dropping rapidly in 1-2 hours (Moss and Scott 1961).

Acclimation effects can occur through many different processes. Chronic exposure to mild hypoxia may allow acclimation of aerobic processes (Heath 1995). Acclimation by increased hemoglobin concentration and hematocrit allowing more oxygen uptake, and decreased metabolism causing decreased oxygen use, both require from one week to one

month (Neill and Bryan 1991). Brief acute exposures to severe hypoxia may have an effect on anaerobic capabilities (Heath 1995). Acclimation may also take place through behavioral processes, as upon exposure to low DO concentrations, the behavior of a nonacclimated fish might use much more energy than that of an acclimated fish, allowing the acclimated fish to conserve energy while being subjected to oxygen stress (Davis 1975).

However, there is also evidence in the literature that some fish do not acclimate to hypoxia. In young striped bass (*Morone saxatilis*), there was little difference in resistance between fish exposed to decreasing oxygen concentrations over 24h and fish exposed to sudden low DO concentrations (Dorfman and Westman 1970). An extended repeat-exposure study with brown trout showed no evidence of acclimation to hypoxia exposure, although DO levels used ranged from 4.0-5.5 ppm, significantly above lethal DO levels (Seager *et al.* 2000). These studies suggest that while acclimation to hypoxia may occur for some fish species, it may be a species-specific phenomenon and that severity of hypoxia may impact any acclimation effect.

Most DO tolerance experiments with fish have found that the range over which DO concentrations affect survival is very narrow. In my experiments at 25°C, 24-hour exposure to DO concentrations from 0.6 to 1.2 ppm O_2, a range of only 0.6 ppm O_2, encompassed the entire range of mortality from 0 to 100%. In their tests on eight different fish species, Downing and Merkens (1957) found that the difference between DO concentrations causing 100% mortality and allowing 100% survival was around 0.5 ppm O_2. Rainbow trout exhibited 100% mortality at 1.7 ppm O_2 and 100% survival at 2.3 ppm O_2 (Magaud 1993). The narrow range between complete mortality and survival in rainbow trout was reaffirmed by Seager *et al*. (2000), who found that range to be at most 1 ppm O_2.

Significant mortality due to hypoxia does not occur in fish until very low DO concentrations are reached. In my experiments, most of the mortality was sustained in both spot and Atlantic menhaden at severely hypoxic DO concentrations of < 1 ppm O_2. For less tolerant species like cutthroat trout, significant mortality has been reported to occur at about 2 ppm O_2 (Wagner *et al.* 2001), while for very tolerant species like roach (*Rutilus rutilus*) significant mortality may not occur until levels drop below 0.5 ppm O_2 (Seager *et al.* 2000). Fish start to use compensating mechanisms like increased ventilation rates (Wannamaker and Rice 2000) at lower DO levels to prevent mortality, but when those are exhausted by conditions exceeding these compensating mechanisms, mortality occurs rapidly.

In my experiments, DO concentrations were dropped from normoxia to the treatment hypoxia concentration in about one hour. I did not have the resources to treat DO reduction rate as a variable, which was acceptable because the DO reduction rate is independent of the mean lethal concentration for Atlantic menhaden, while it may change time to mortality (Burton *et al.* 1980). DO concentrations were dropped rapidly to prevent any rapid acclimation response to hypoxia of which the fish might be capable which could confound results. Rapid DO reduction also simulates the field conditions likely to cause mortality, as direct mortality is most likely to occur in the field when DO concentrations decrease very rapidly, and fish are cornered or other conditions confuse escape behavior (Breitburg 2002). DO concentrations can drop quickly in the summer, with decreases of 6 ppm in 4 hours and 1 ppm in 14 minutes possible in Chesapeake Bay (Breitburg 1990).

Conclusions

My results suggest that direct effects of hypoxia are not likely a major source of mortality for juvenile spot and Atlantic menhaden. While hypoxic conditions can be spatially and temporally pervasive in estuaries in the summer, they are typically constrained to the bottom waters, often leaving well-oxygenated habitat available in which fish can survive. Direct effects of hypoxia may be reduced due to the ability of fish to avoid hypoxic areas. Field studies find much lower fish densities when DO is < 2 ppm (Howell and Simpson 1994, Eby and Crowder 2002). Eby and Crowder (2002) found the presence-absence threshold in the Neuse River Estuary to be 2.3 ppm O_2 for spot and 2.6 ppm O_2 for Atlantic menhaden, significantly higher than the DO concentrations I found necessary to cause mortality in either species, suggesting that both Atlantic menhaden and spot will often avoid areas with hypoxic concentrations severe enough to cause mortality. Laboratory trials support the ability of fish to avoid hypoxia, as many species, including juvenile spot and Atlantic menhaden, avoided 1 ppm O_2 (Wannamaker and Rice 2000) and Southern flounder began to choose well-oxygenated water at 3.7 mL/L O_2 (Deubler and Posner 1963). The typical availability of well-oxygenated habitat to use as a refuge suggests that direct effects of hypoxia may not be a significant source of mortality for many estuarine species.

Sublethal effects of hypoxia are potentially greater than the direct effects. Fish generally feed less when in hypoxic conditions (Kramer 1987), which can lead to a decreased growth rate. Laboratory studies found that growth and food consumption increased as DO concentrations increased (Stewart *et al.* 1967, Whitworth 1968, Bejda *et al.* 1992, McNatt 2002). Field studies find reduced fish size and biomass in hypoxic areas (Howell and Simpson 1994), suggesting that reduced growth may be caused by hypoxic stress. Long-term

(12-week) hypoxia exposure to 1 ppm O_2 impaired reproduction in the common carp (*Cyprinus carpio*) (Wu *et al.* 2003). Hypoxia may increase competition or predation risk due to compressed habitat, increased species overlap, and resulting increased vulnerability and encounter rates (Breitburg *et al.* 1999, Eby and Crowder 2002).

Increases in the spatial or temporal extent of hypoxia could increase the direct and indirect effects of hypoxia currently experienced by fishes. Hypoxia is increasing in frequency, severity, and area both locally and globally (Diaz and Rosenberg 1995, Rabalais 2001, Eby and Crowder 2002). This increase in hypoxia events is primarily due to increased nutrient inputs from intensive farming, fertilizer application, deforestation, and domestic wastewater (Wu 2002). Hypoxia in estuaries is important because many commercially viable fisheries, including Atlantic menhaden and spot, depend on estuarine habitat for critical life stages such as spawning or recruitment (Mallin *et al.* 2000). The eutrophication that causes many hypoxic events may actually increase productivity enough to more than offset any diminished abundance caused by hypoxia (Breitburg 2002). However, productivity benefits likely cease at some point and are replaced with environmental problems that cause decreases in landings quantity or quality (Rabalais *et al*. 2002).

A full understanding of the effects of hypoxia on fish populations requires an approach that integrates lethal and sublethal effects, direct and indirect effects, and fish behavior. My results will be incorporated into a spatially-explicit, individual-based model using information about mortality, growth, and behavioral avoidance of hypoxia for several juvenile estuarine-dependent species to describe potential responses of estuarine populations to hypoxia. This approach will allow evaluation of the relative importance of various factors and evaluation of the effects of hypoxia on growth, survival, and fish production.

Table 1. Summary of observed tolerances to hypoxia for Atlantic menhaden and spot. (LC_{05} = concentration causing 5% mortality; LC_{50} = concentration causing 50% mortality)

Species	Information	Source
Atlantic menhaden (*Brevoortia tyrannus*)	Oxygen reduction rates had no effect on absolute oxygen concentration causing death (~ 0.4 ppm O_2), although faster reduction resulted in faster time to death	
	2h LC_{05} of 1.00 ppm O_2; 96h LC_{05} of 1.55 ppm O_2	Burton *et al.* 1980
	2h LC_{50} of 0.70 ppm O_2; 96h LC_{50} of 1.04 ppm O_2	
	Lethal threshold concentration (DO concentration at which LC_{50} becomes constant independent of exposure time) estimated ~ 1.1 ppm O_2	
	Absolute DO more important than rate of DO reduction (6.0 to 0 ppm O_2 over 6, 12, 24, and 48h); all rates resulted in mortality at about 0.4 ppm O_2 at 28-30°C	Richardson *et al.* 1975
	100% survival at 1.5 ppm O_2 for 2 weeks	McNatt 2002
	Absent in Cape Fear River collections < 1.4 ppm O_2	Schwartz *et al.* 1981
	Avoidance threshold of 2.6 ppm O_2 from field data	Eby and Crowder 2002
Spot (*Leiostomus xanthurus*)	1h LC_{05} of 0.56 ppm O_2; 96h LC_{05} of 0.81 ppm O_2	
	1h LC_{50} of 0.49 ppm O_2; 96h $LC_{50}$of 0.70 ppm O_2	Burton *et al.* 1980
	Lethal threshold concentration ~ 0.7 ppm O_2	
	Died at 0.82 ppm O_2 at 25ºC and 17-21ppt salinity	Subrahmanyam 1980
	100% survival at 1.5 ppm O_2 for 2 weeks	McNatt 2002
	0% mortality after 4 days at 1.8-2.7 ppm O_2/L	
	Died within 4h at 11-13% saturation (0.8-1.0 ppm O_2) at 25°C and 18-20ppt salinity	Pihl *et al.* 1991
	Died within 4h when dropped below 1.4 ppm O_2	
	Absent in York River trawls below 2 ppm O_2	
	Absent in Cape Fear River collection < 1.0 ppm O_2	Schwartz *et al.* 1981
	Avoidance threshold of 2.3 ppm O_2 from field data	Eby and Crowder 2002

Table 2. Mean and standard deviation (SD) of DO measurements for each DO treatment pooled across all trials.

DO Treatment (ppm)	Mean ± 1 SD (n)
0.6	0.64 ± 0.144 (174)
0.9	0.91 ± 0.154 (818)
1.2	1.21 ± 0.147 (1182)
0.6 Acclimated	0.59 ± 0.076 (130)

Table 3. Cox regression parameter estimates for Atlantic menhaden and spot.

Atlantic menhaden

Analysis of Maximum Likelihood Estimates

Variable	DF	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq	Hazard Ratio
Mass (g)	1	0.07972	0.01861	18.3481	<0.0001	1.083
DO Concentration (0.1 ppm)	1	-0.65621	0.02649	613.5116	<0.0001	0.519
Temperature (ºC)	1	0.21447	0.02327	84.9238	<0.0001	1.239

Spot

Analysis of Maximum Likelihood Estimates

Variable	DF	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq	Hazard Ratio
Mass (g)	1	-0.04558	0.01548	8.6729	0.0032	0.955
DO Concentration (0.1 ppm)	1	-0.65430	0.02437	720.7344	<0.0001	0.520
Temperature (ºC)	1	0.1038	0.01655	39.3175	<0.0001	1.109

Table 4. Mean and percentile mass (g) for Atlantic menhaden and spot.

	Atlantic Menhaden		Spot	
	25°C	**30°C**	**25°C**	**30°C**
10%	2.46	5.43	3.77	1.98
50%	3.34	6.90	5.14	5.11
Mean	4.40	7.48	6.36	5.26
90%	5.75	10.37	8.13	8.01

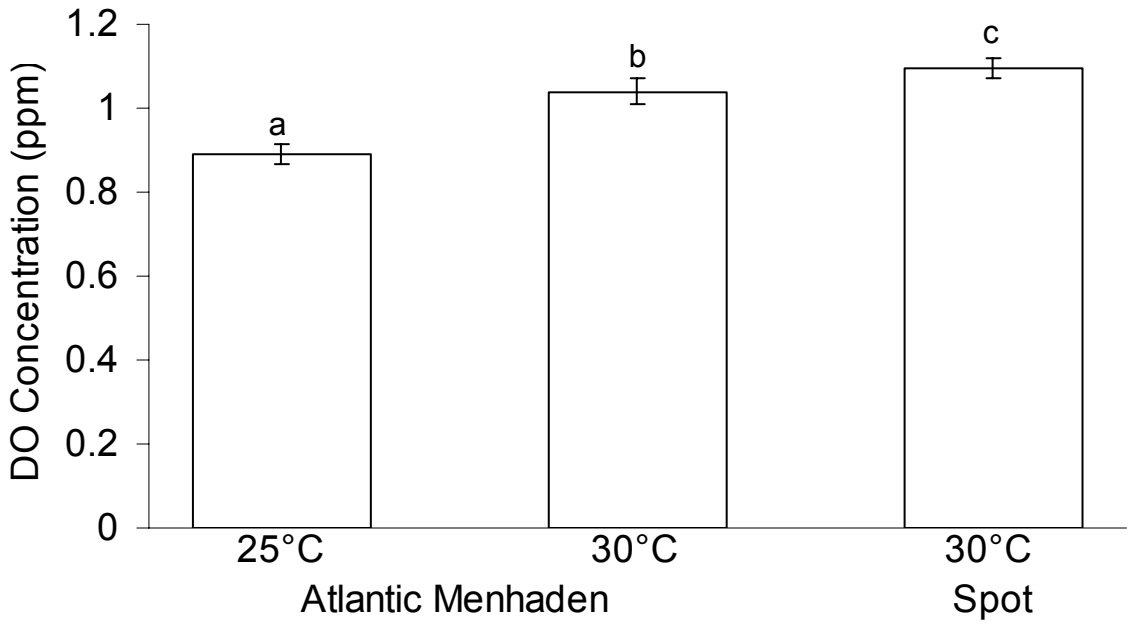


Figure 1. LC_{50} DO concentrations for Atlantic menhaden at 25 and 30°C and spot at 30°C; error bars show 95% confidence intervals. Bars labeled with different letters are significantly different ($P<0.05$).

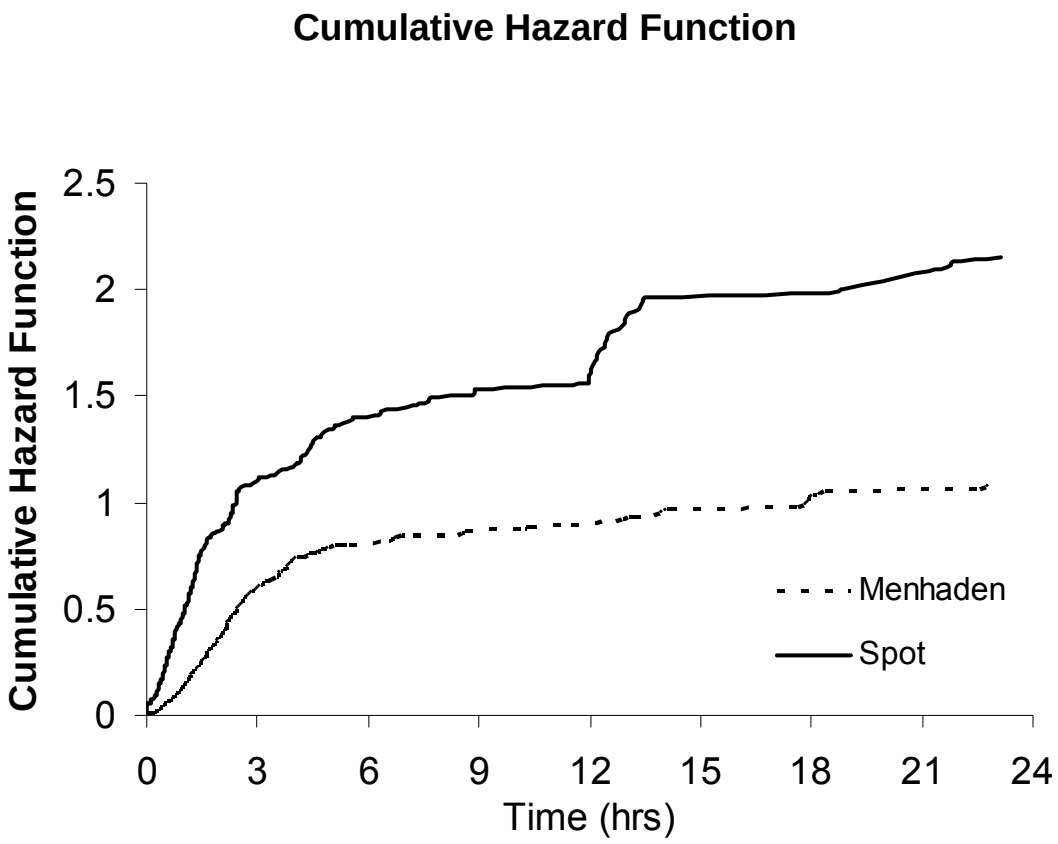
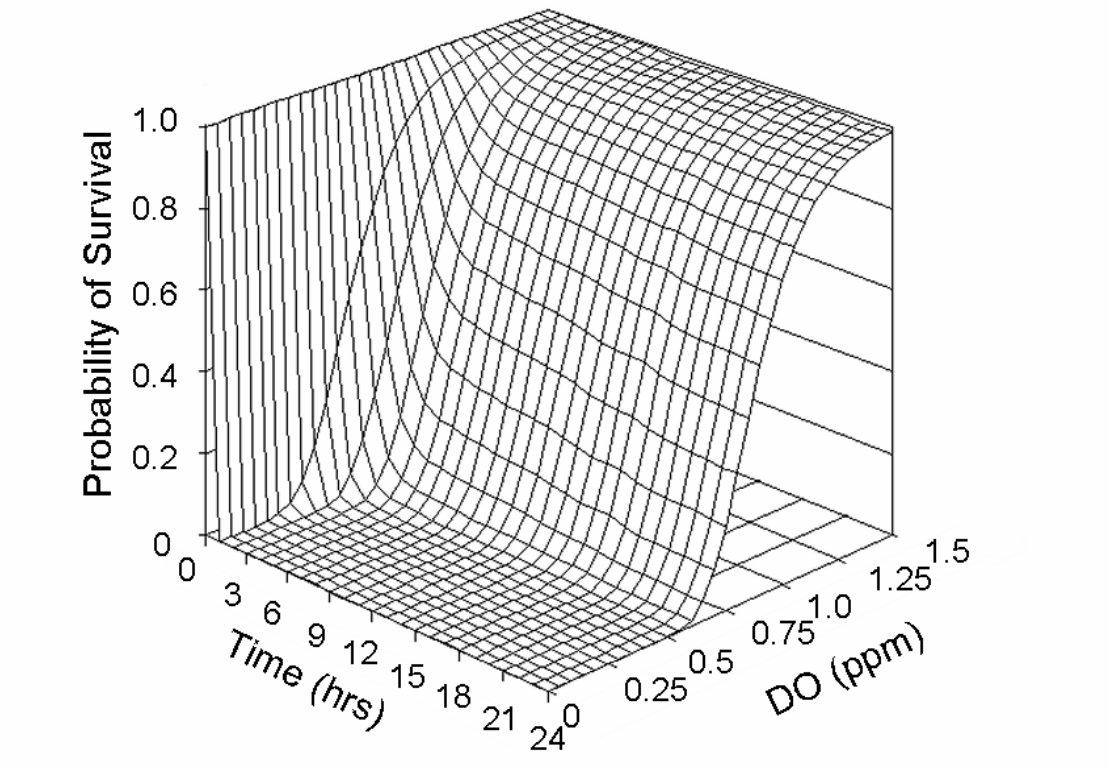


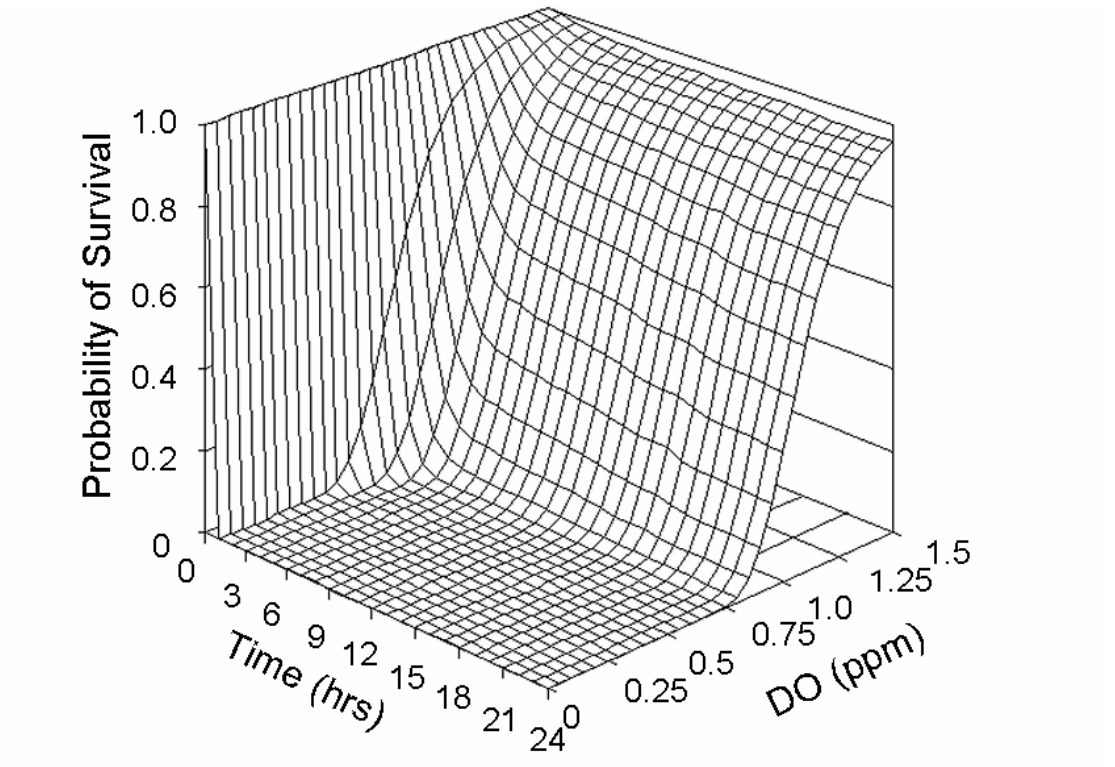
Figure 2. Cumulative hazard functions for Atlantic menhaden and spot. Functions are illustrated for average fish size and conditions, i.e., a 5.99g Atlantic menhaden exposed to 0.91 ppm O_2 at 27.6°C and a 5.82g spot exposed to 0.93 ppm O_2 at 27.5°C.

Figure 3. Response surfaces produced from Cox regression models showing the relationship of probability of survival to DO concentration and exposure time for each species-temperature combination tested: a) Atlantic menhaden at 25°C, b) Atlantic menhaden at 30°C, c) spot at 25°C, and d) spot at 30°C. Mean mass was used for each species at each temperature (Table 4).

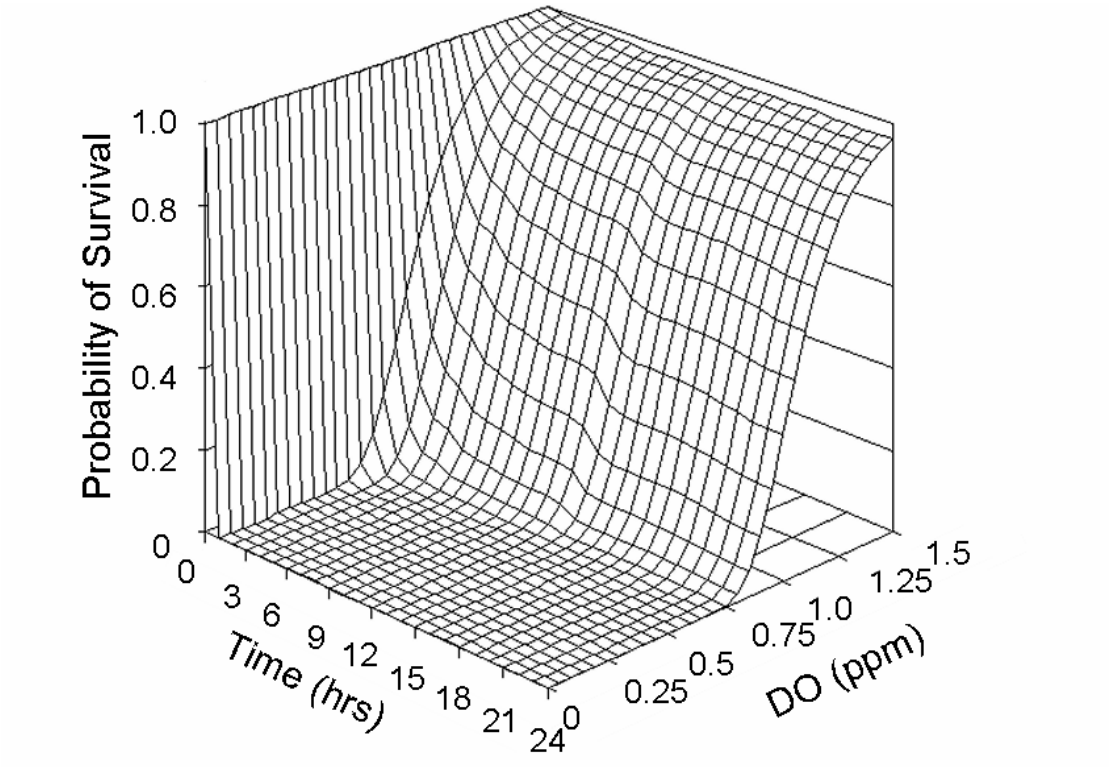
a) **Atlantic Menhaden at 25°C**



b) **Atlantic Menhaden at 30°C**



c) **Spot at 25°C**



d) **Spot at 30°C**

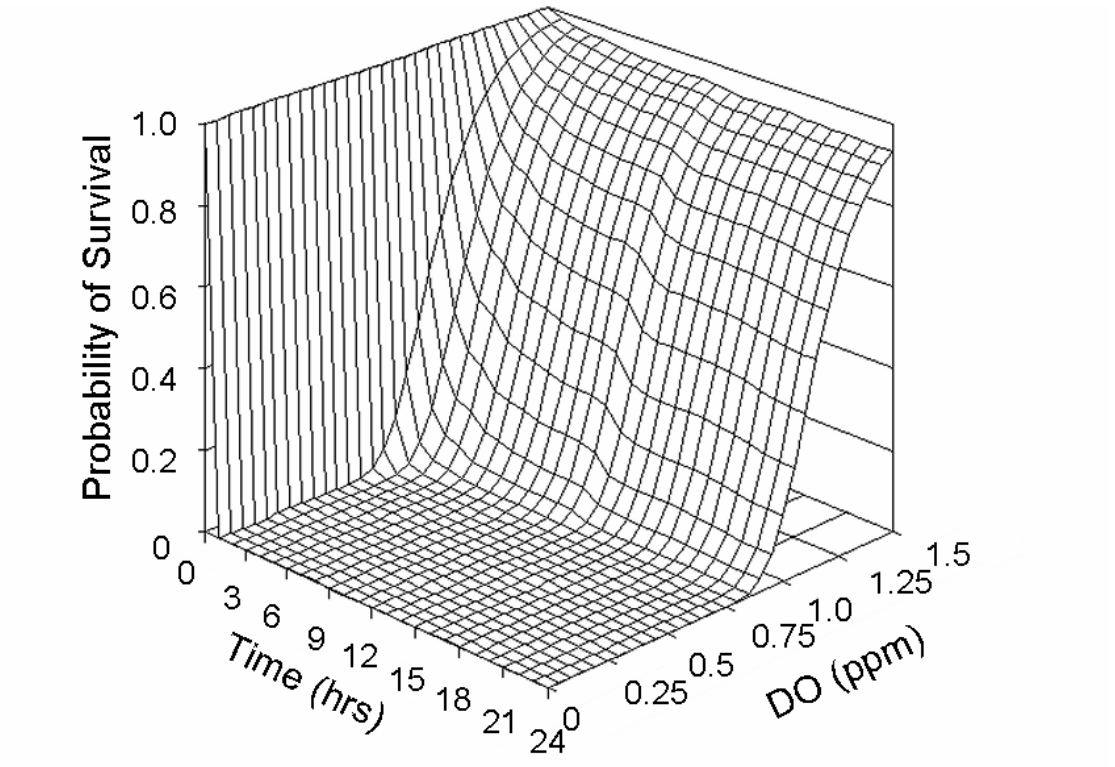
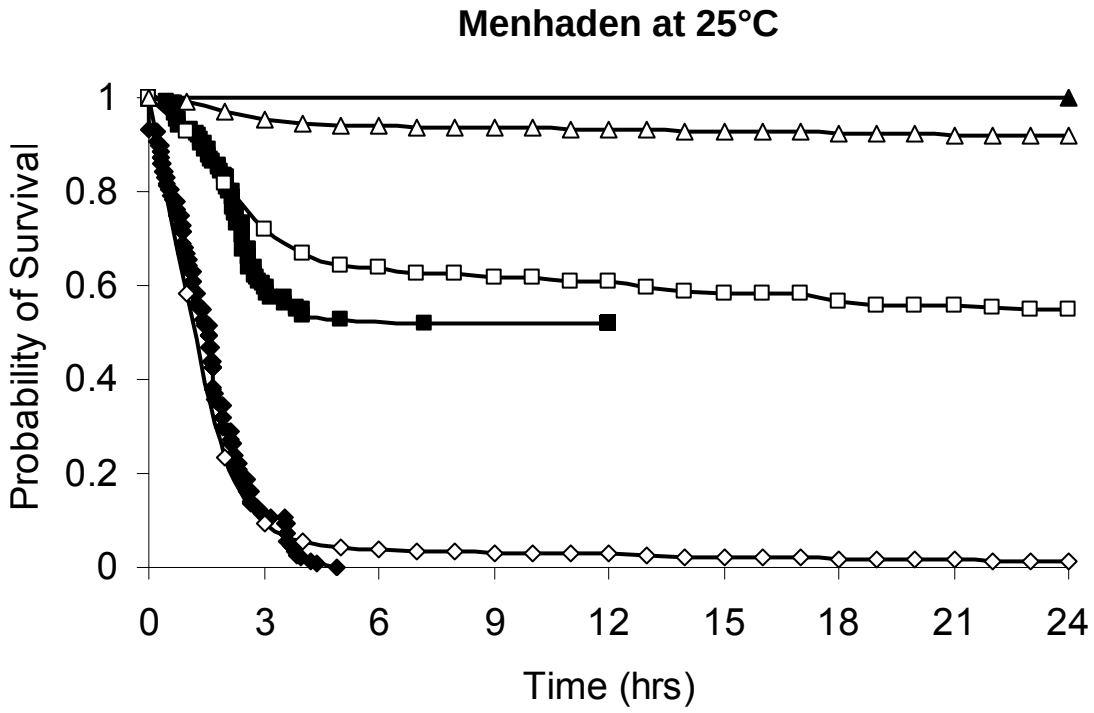
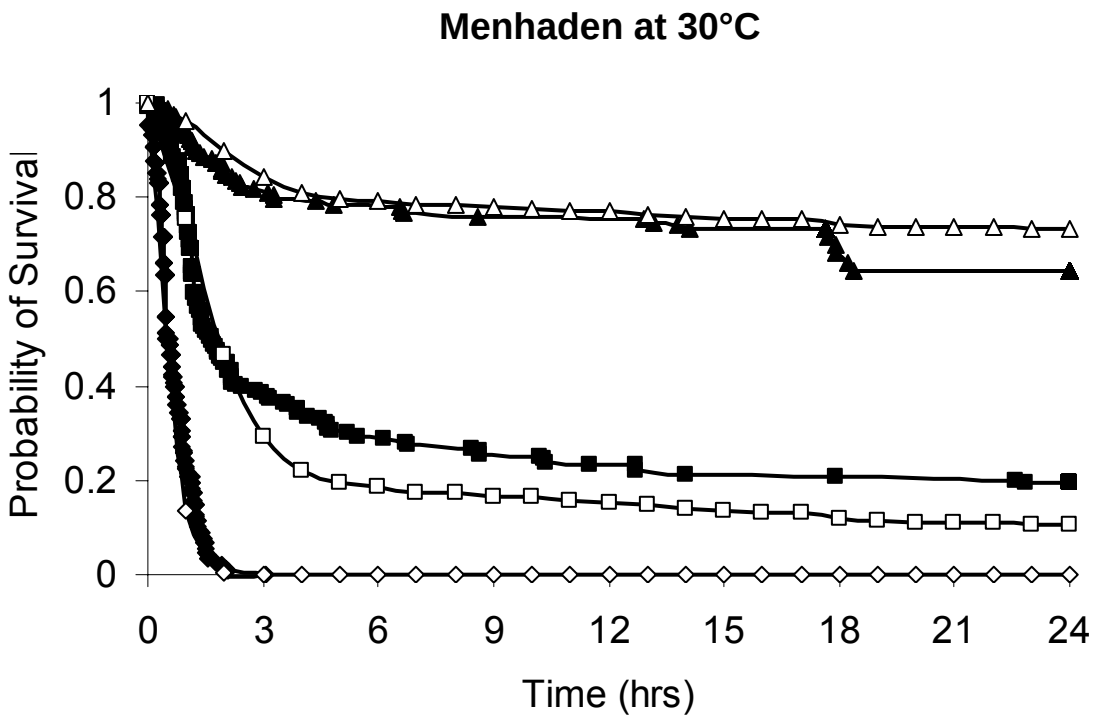


Figure 4. Visual examination of model fit by comparison of Cox regression model predictions to Kaplan-Meier estimates for each species-temperature combination tested: a) Atlantic menhaden at 25°C, b) Atlantic menhaden at 30°C, c) spot at 25°C, and d) spot at 30°C. Closed symbols indicate Kaplan-Meier estimates and open symbols indicate Cox regression model predictions. The symbol shape denotes the DO treatment: ♦ = 0.6 ppm, ■ = 0.9 ppm, and ▲ = 1.2 ppm. Mean mass was used for each species at each temperature (Table 4).

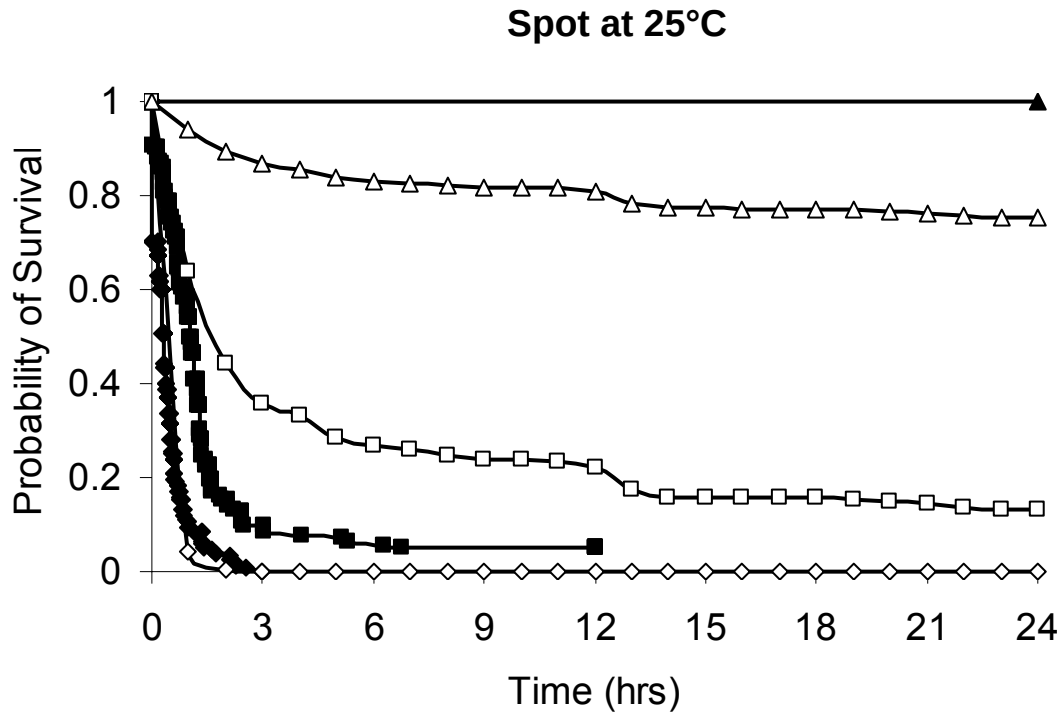
a)


Menhaden at 25°C
Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

b)


Menhaden at 30°C
Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

c)


Spot at 25°C
Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
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6
9
12
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18
21
24
Time (hrs)

d)

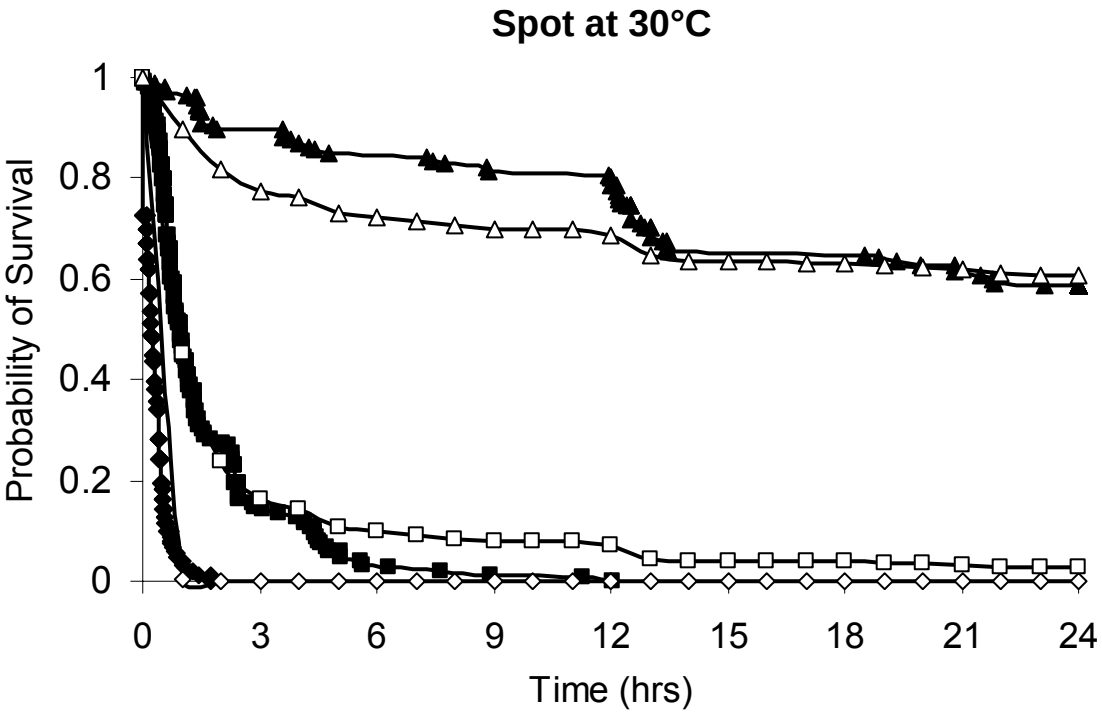
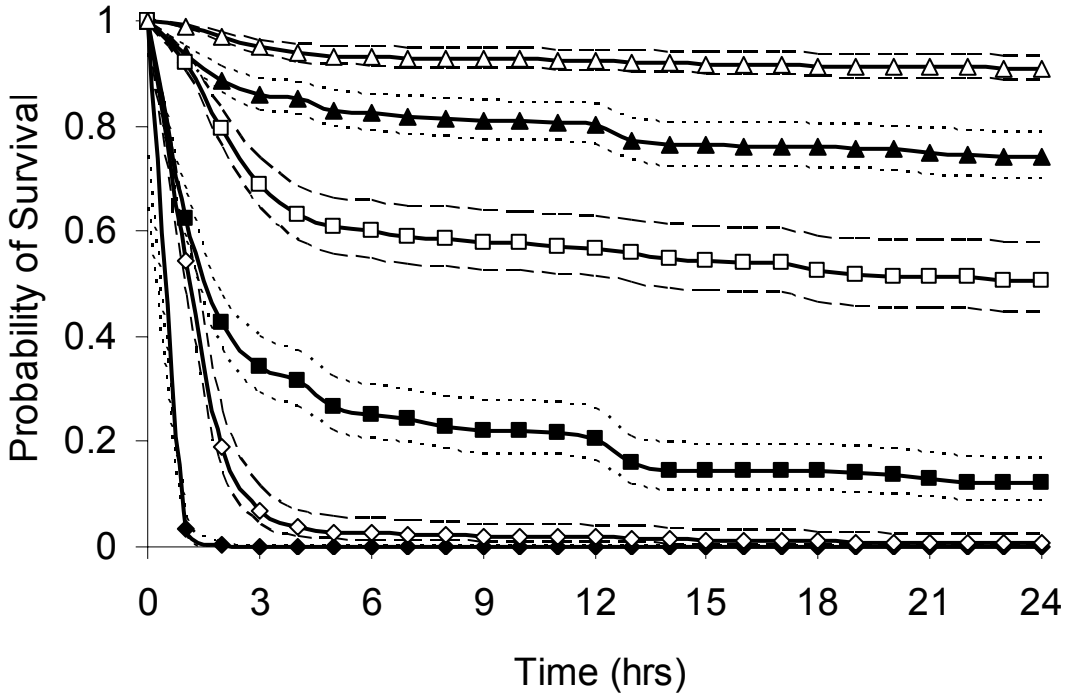

Spot at 30°C
Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

Figure 5. Differences in hypoxia tolerance of Atlantic menhaden and spot at both temperatures tested, a) 25°C and b) 30°C. Closed symbols indicate spot and open symbols indicate Atlantic menhaden. The symbol shape denotes the DO treatment: ♦ = 0.6 ppm, ■ = 0.9 ppm, and ▲ = 1.2 ppm. Dotted lines represent 95% confidence intervals. Mean mass was used for Atlantic menhaden (5.99g) and spot (5.82g).

a) **25°C**


Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

b) **30°C**

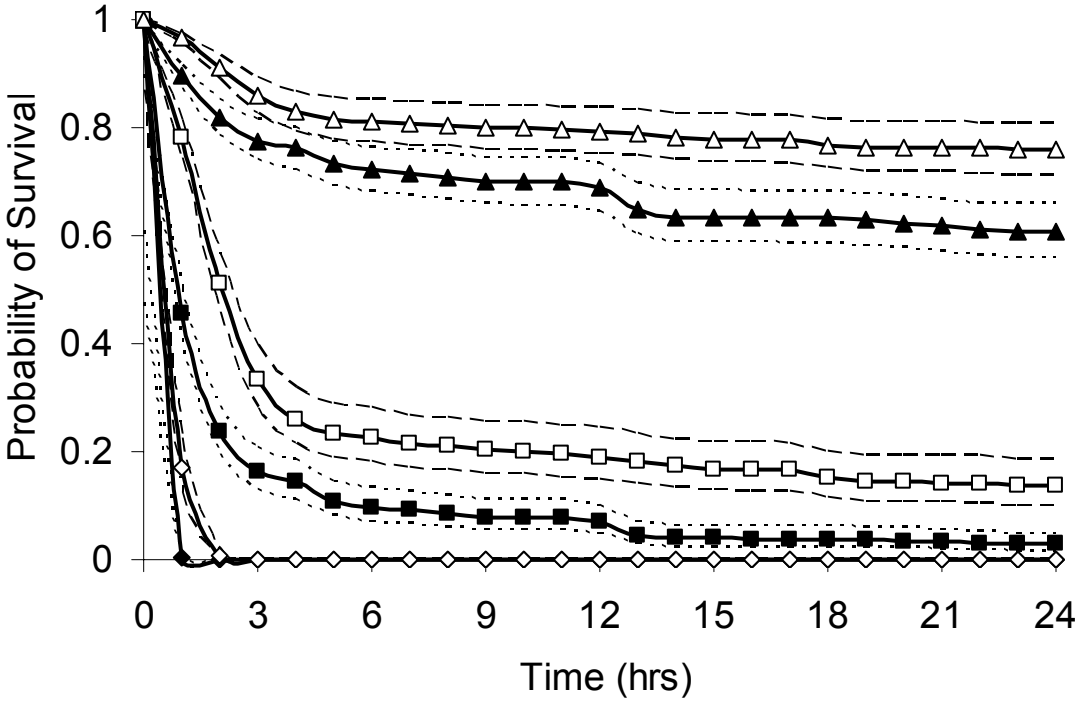
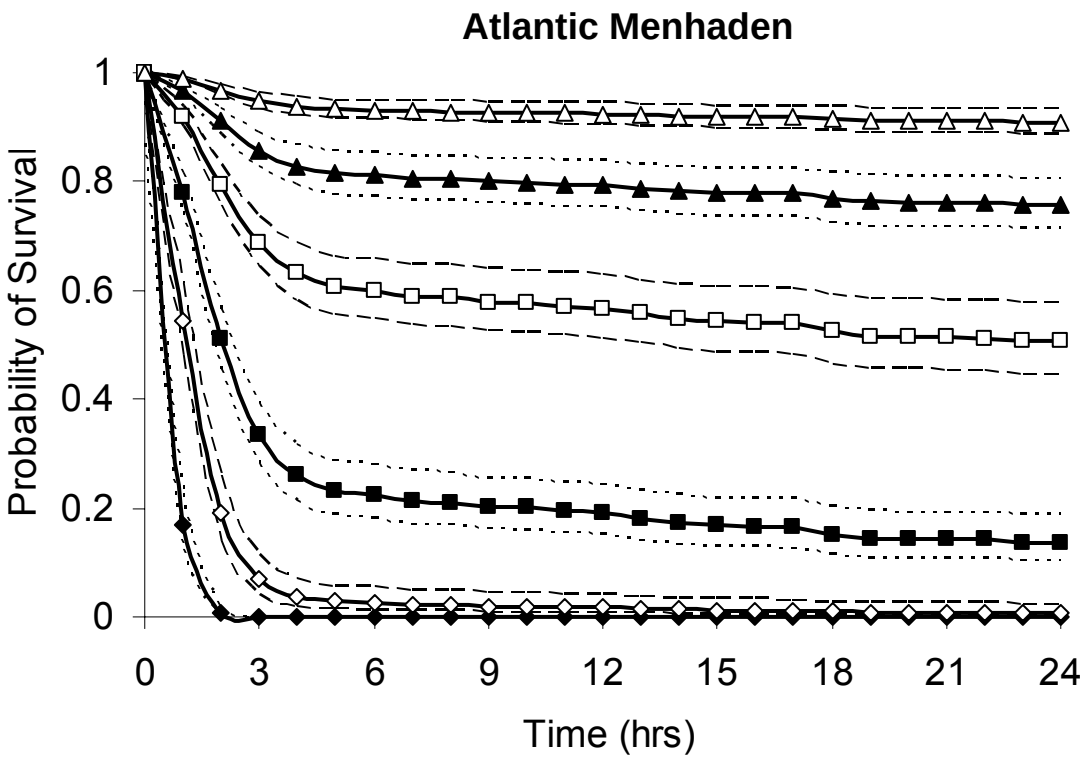

Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

Figure 6. Differences in hypoxia tolerance at 25°C and 30°C for both species tested, a) Atlantic menhaden and b) spot. For figures a-b) closed symbols indicate 30°C and open symbols indicate 25°C. The symbol shape denotes the DO treatment: ♦ = 0.6 ppm, ■ = 0.9 ppm, and ▲ = 1.2 ppm. Dotted lines represent 95% confidence intervals. Mean mass was used for Atlantic menhaden (5.99g) and spot (5.82g).

a)


Atlantic Menhaden
Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

b)

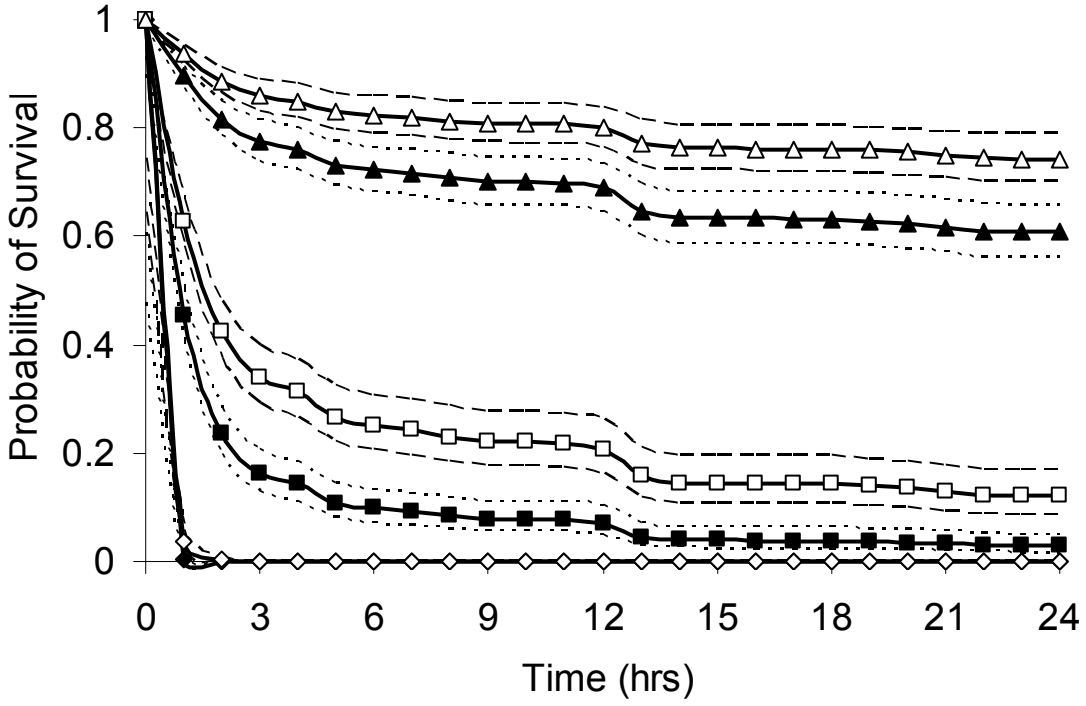
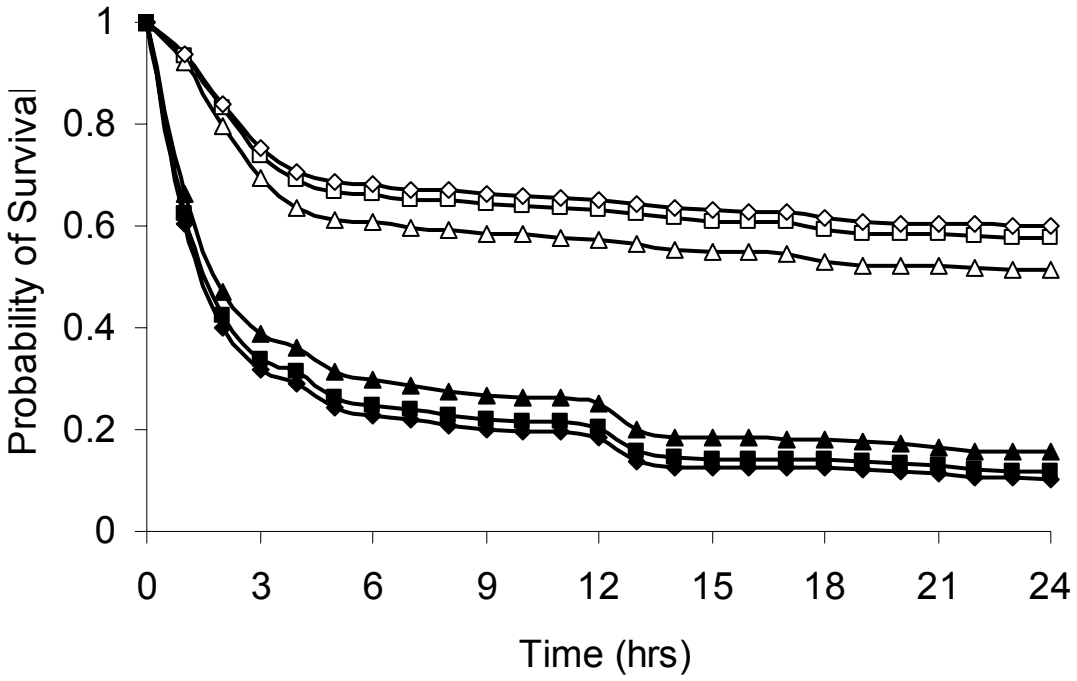
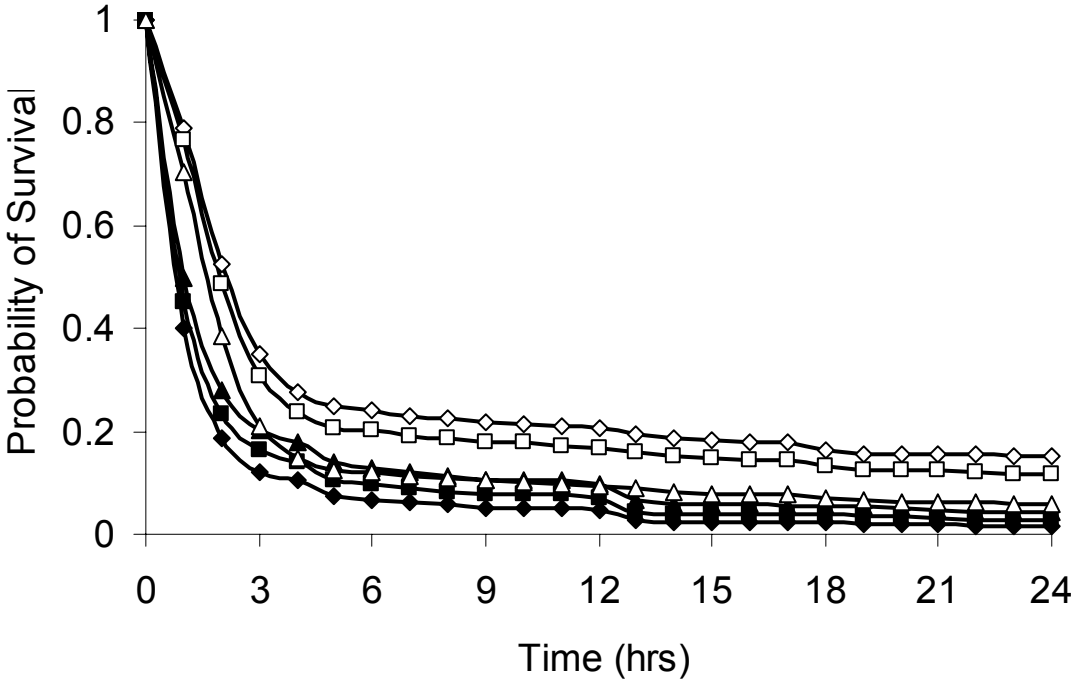

Spot
Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

Figure 7. Differences in hypoxia tolerance of spot and Atlantic menhaden to 0.9 ppm O_2 at 10^{th}, 50^{th} and 90^{th} mass percentiles (Table 4) for both temperatures tested, a) 25°C and b) 30°C. Closed symbols indicate spot and open symbols indicate Atlantic menhaden. Symbol shape denotes the fish mass: ♦ = 10^{th} percentile, ■ = 50^{th} percentile and ▲ = 90^{th} percentile.

a) **25°C**


Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

b) **30°C**


Probability of Survival
1
0.8
0.6
0.4
0.2
0
0
3
6
9
12
15
18
21
24
Time (hrs)

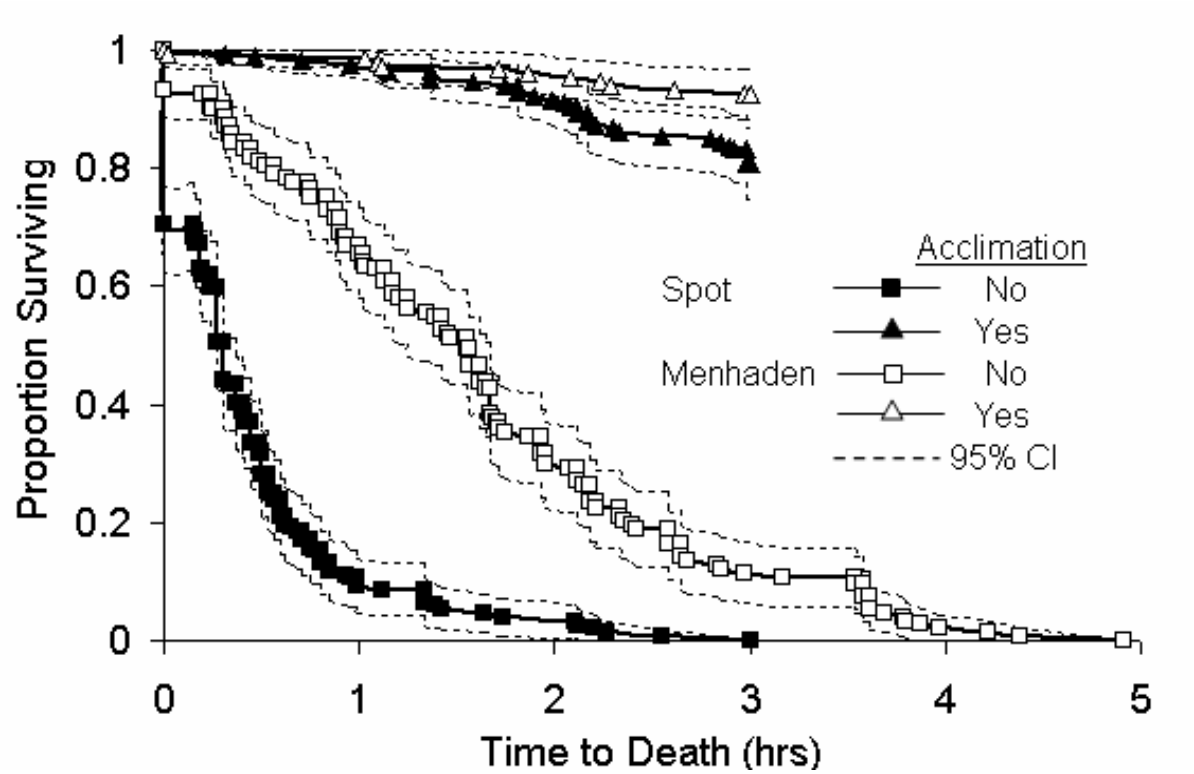


Figure 8. Effect of acclimation on mortality response to hypoxia. Proportion survival and 95% confidence intervals estimated using the Kaplan-Meier method. The acclimation effect was tested at 0.6 ppm O_2 and 25°C.

Literature Cited

Able, K.W. 1999. Measures of juvenile fish habitat quality: examples from a National Estuarine Research Reserve. American Fisheries Society Symposium 22: 134-147.

Alabaster, J.S., D.W.M. Herbert, and J. Hemens. 1957. The survival of rainbow trout (*Salmo gairdnerii* Richardson) and perch (*Perca fluviatilis* L.) at various concentrations of dissolved oxygen and carbon dioxide. Annals of Applied Biology 45: 177-188.

Allison, P.D. 1995. Survival Analysis Using the SAS® System: A Practical Guide. SAS Institute, Inc.: Cary, NC. 292 pp.

Atwood, D.K., A. Bratkovitch, M. Gallagher, and G.L. Hitchcock, eds. 1994. Dedicated Issue on NOAA's Nutrient Enhanced Coastal Ocean Productivity Study. Estuaries 17(4).

Bejda, A., B. Phelan, and A. Studholme. 1992. The effect of dissolved oxygen on growth of young-of-year winter flounder, (*Pseudopleuronectes americanus*). Environmental Biology of Fishes 34: 321-327.

Borsuk, M., R. Clemen, L. Maguire, and K. Reckhow. 2001. Stakeholder values and scientific modeling in the Neuse River watershed. Group Decision and Negotiation 10(4): 355-373.

Breitburg, D.L. 2002. Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. Estuaries 25(4b): 767-781.

Breitburg, D.L., K.A. Rose, and J.H. Cowan. 1999. Linking water quality to larval survival: predation mortality of fish larvae in an oxygen-stratified water column. Marine Ecology Progress Series 178: 39-54.

Breitburg, D.L., T. Loher, C.A. Pacey, and A. Gerstein. 1997. Varying effects of low dissolved oxygen on trophic interactions in an estuarine food web. Ecological Monographs 67(4): 489-507.

Breitburg, D.L. 1994. Behavioral response of fish larvae to low dissolved oxygen concentrations in a stratified water column. Marine Biology 120: 615-625.

Breitburg, D.L. 1992. Episodic hypoxia in Chesapeake Bay: Interacting effects of recruitment, behavior, and physical disturbance. Ecological Monographs 62(4): 525-546.

Breitburg, D.L. 1990. Near-shore hypoxia in the Chesapeake Bay: patterns and relationships among physical factors. Estuarine, Coastal, and Shelf Science 30: 593-609.

Brett, J.R. and N.R. Glass. 1973. Metabolic rates and critical swimming speeds of sockeye salmon (*Oncorhynchus nerka*) in relation to size and temperature. Journal of the Fisheries Research Board of Canada 30: 379-387.

Burkholder, J.M., M.A. Mallin, and H.B. Glasgow. 1999. Fish kills, bottom-water hypoxia, and the toxic Pfiesteria complex in the Neuse River and Estuary. Marine Ecology Progress Series 179: 301-310.

Burton, D.T., L.B. Richardson, and C.J. Moore. 1980. Effect of oxygen reduction rate and constant low dissolved oxygen concentrations on two estuarine fish. Transactions of the American Fisheries Society 109: 552-557.

Collett, D. 1994. Modelling survival data in medical research, 1st ed. Chapman and Hall: New York. 347 pp.

Copeland, B.J. and J. Grey. 1989. Albemarle-Pamlico Estuarine System: Preliminary Analysis of the Status and Trends. Albemarle-Pamlico Study Report 89-13A: North Carolina Department of Environment and Natural Resources, Raleigh, NC.

Cox, D.R. 1975. Partial likelihood. Biometrika 62: 269-276.

Cox, D.R. 1972. Regression models and life-tables. Journal of the Royal Statistical Society, Series B 34: 187-220.

Davis, J.C. 1975. Minimal dissolved oxygen requirements of aquatic life with emphasis on Canadian species: a review. Journal of the Fisheries Research Board of Canada 32: 2295-2332.

Deubler, E.E. and G.S. Posner. 1963. Response of postlarval flounders, *Paralichthys lethostigma*, to water of low oxygen concentration. Copeia 2: 312-317.

Diaz, R.J. and R. Rosenberg. 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. Oceanography and Marine Biology: An Annual Review 33: 245-303.

Dixon, P.M. and M.C. Newman. 1991. Analyzing toxicity data using statistical models for time-to-death: an introduction, pp. 207-242. *In*: M.C. Newman and A.W. McIntosh (eds.), Metal Ecotoxicology: Concepts and Applications. Lewis Publishers: Chelsea, Michigan.

Dorfman, D. and J. Westman. 1970. Responses of some anadromous fishes to varied oxygen concentrations and increased temperatures. New Jersey Water Resources Research Institute, Project B-012-N1. The State University of Rutgers: New Brunswick, New Jersey. *In*: Burton, D.T., L.B. Richardson, and C.J. Moore. 1980. Effect of oxygen reduction rate and constant low dissolved oxygen concentrations on two estuarine fish. Transactions of the American Fisheries Society 109: 552-557.

Downing, K.M. and J.C. Merkens. 1957. The influence of temperature on the survival of several species of fish in low tensions of dissolved oxygen. Annals of Applied Biology 45: 261-267.

Durbin, A.G., E.G. Durbin, P.G. Verity, and T.J. Smayda. 1981. Voluntary swimming speeds and respiration rates of a filter-feeding planktivore, the Atlantic menhaden, *Brevoortia tyrannus* (Pisces: Clupeidae). Fishery Bulletin 78(4): 877-886.

Eby, L.A. and L.B. Crowder. 2002. Hypoxia-based habitat compression in the Neuse River Estuary: context-dependent shifts in behavioral avoidance thresholds. Canadian Journal of Fisheries and Aquatic Sciences 59: 952-965.

Friedland, K.D., D.W. Ahrenholz, and J.F. Guthrie. 1996. Formation and seasonal evolution of Atlantic menhaden juvenile nurseries in coastal estuaries. Estuaries 19(1): 105-114.

Heath, A.G. 1995. Water Pollution and Fish Physiology, 2nd ed. Lewis Publishers: Boca Raton, Florida. 359 pp.

Howell, P. and D. Simpson. 1994. Abundance of marine resources in relation to dissolved oxygen in Long Island Sound. Estuaries 17(2): 394-402.

Hughes, G.M. 1984. Scaling of respiratory areas in relation to oxygen consumption of vertebrates. Experientia 40(6): 519-652.

Hunn, J. and R. Schnick. 1990. Toxic substances. *In*: F. Meyer and L. Barclay (eds.), Field Manual for the Investigation of Fish Kills. Fish and Wildlife Service Resource Publication 177. United States Department of the Interior: Washington, D.C.

Kramer, D.L. 1987. Dissolved oxygen and fish behavior. Environmental Biology of Fishes 18(2): 81-92.

Kramer, G.L. 1975. Studies on the lethal dissolved oxygen levels for young brown shrimp, *Penaeus aztecus*. Proceedings of the Annual Meeting, World Mariculture Society 6: 157-167. *In*: Allan, G.L. and G.B. Maguire. 1991. Lethal levels of low dissolved oxygen and effects of short-term oxygen stress on subsequent growth of juvenile *Penaeus monodon*. Aquaculture 94: 27-37.

Magaud, H., B. Migeon, P. Morfin, J. Garric, and E. Vindimian. 1997. Modelling fish mortality due to urban storm run-off: interacting effects of hypoxia and un-ionized ammonia. Water Research 31(2): 211-218.

Magaud, H. 1993. Effet létal d'une hypoxie en présence d'ammoniaque sur les truitelles arc-ec-ciel. Rapport technique. Cemagref, Lyon, France, p. 31. *In*: Seager, J., I. Milne, M. Mallet, and I. Sims. 2000. Effects of short-term oxygen depletion on fish. Environmental Toxicology and Chemistry 19(12): 2937-2942.

Mallin, M.A., J.M. Burkholder, L.B. Cahoon, and M.H. Posey. 2000. North and South Carolina coasts. Marine Pollution Bulletin 41: 56-75.

McCullagh, P. and J.A. Nelder. 1989. Generalized Linear Models, 2nd ed. Chapman and Hall: New York. 511 pp.

McNatt, R.A. 2002. Hypoxia-induced growth rate reduction in two juvenile estuary-dependent fishes. M.S. Thesis. North Carolina State University: Raleigh, NC.

Minello, T.J. 1999. Nekton densities in shallow estuarine habitats of Texas and Louisiana and the identification of essential fish habitat. American Fisheries Society Symposium 22: 43-75.

Moss, D.D. and D.C. Scott. 1961. Dissolved oxygen requirements of three species of fish. Transactions of the American Fisheries Society 90: 377-393.

Moyle, P. and J. Cech. 2000. Fishes: An Introduction to Ichthyology, 4th ed. Prentice Hall: Uppersaddle River, New Jersey. 612 pp.

NC DMF (North Carolina Division of Marine Fisheries). 1999. Stock Status of Important Coastal Fisheries in North Carolina, 1999. www.ncdmf.net/stocks. NC DMF: Morehead City, NC.

NC DWQ (North Carolina Division of Water Quality). 2003. Fish Kill Event Update. www.esb.enr.state.nc.us/Fishkill/fishkillmain.htm. NC DWQ: Raleigh, NC.

Neill, W.H. and J.D. Bryan. 1991. Responses of fish to temperature and oxygen, and response integration through metabolic scope. Aquaculture and Water Quality 3: 30-57.

Newman, M.C. and P.M. Dixon. 1996. Ecologically meaningful estimates of lethal effect in individuals, pp. 225-253. *In*: M.C. Newman and C.H. Jagoe (eds.), Ecotoxicology: A Hierarchical Treatment. CRC/Lewis Press: Chelsea, Michigan.

Newman, M.C. and J.T. McCloskey. 1996. Time-to-event analyses of ecotoxicity data. Ecotoxicology 5: 187-196.

Newman, M.C. 1995. Quantitative Methods in Aquatic Ecotoxicology. CRC Press: Boca Raton, Florida. 426 pp.

Newman, M.C. and M.S. Aplin. 1992. Enhancing toxicity data interpretation and prediction of ecological risk with survival time modeling: an illustration using sodium chloride toxicity to mosquitofish (*Gambusia holbrooki*). Aquatic Toxicology 23: 85-96.

Paerl, H.W., J.L. Pinckney, J.M. Fear, and B.L. Peierls. 1999. Fish kills and bottom-water hypoxia in the Neuse River and Estuary: reply to Burkholder *et al.* Marine Ecology Progress Series 186: 307-309.

Paerl, H.W., J.L. Pinckney, J.M. Fear and B.L. Peierls. 1998. Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA. Marine Ecology Progress Series 166: 17-25.

Pihl, L.S., P. Baden, and R.J. Diaz. 1991. Effects of periodic hypoxia on distribution of demersal fish and crustaceans. Marine Biology 108: 349-360.

Plante, S., D. Chabot, and J.-D. Dutil. 1998. Hypoxia tolerance in Atlantic cod. Journal of Fish Biology 53: 1342-1356.

Rabalais, N.N., R.E. Turner, and W.J. Wiseman. 2002. Gulf of Mexico hypoxia, a.k.a. "The Dead Zone". Annual Review of Ecology and Systematics 33: 235-263.

Rabalais, N.N. 2001. The effects of seasonally severe hypoxia on continental shelf benthos. Presented at the 6th International Symposium on Fish Physiology, Toxicology, and Water Quality. La Paz, Mexico, 22-26 January 2001. *In*: Wu, R.S.S. 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Pollution Bulletin 45: 35-45.

Richardson, L.B., S.L. Margrey, and D.T. Burton. 1975. The effects of several rates of hypoxic stress on the mortality of Atlantic menhaden. Assoc. Southeastern Biological Bulletin 22 (2).

Rose, K.A. 2000. Why are quantitative relationships between environmental quality and fish populations so elusive? Ecological Applications 10: 367-385.

SAS OnlineDoc V8. 1999. www.ncsu.edu/it/sas/help. SAS Institute, Inc.: Cary, NC.

Schmitten, R.A. 1999. Essential fish habitat: opportunities and challenges for the next millennium. American Fisheries Society Symposium 22: 3-10.

Schurmann, H. and J.F. Steffensen. 1992. Lethal oxygen levels at different temperatures and the preferred temperature during hypoxia of the Atlantic cod, *Gadus morhua* L. Journal of Fish Biology 41: 927-934.

Schwartz, F.J., W.T. Hogarth, and M.P. Weinstein. 1981. Marine and freshwater fishes of the Cape Fear estuary, North Carolina, and their distribution in relation to environmental factors. Brimleyana 7: 17-37.

Seager, J., I. Milne, M. Mallet, and I. Sims. 2000. Effects of short-term oxygen depletion on fish. Environmental Toxicology and Chemistry 19(12): 2937-2942.

Secor, D.H. and T.E. Gunderson. 1998. Effects of hypoxia and temperature on survival, growth, and respiration of juvenile Atlantic sturgeon (*Acipenser oxyrinchus*). Fishery Bulletin 96: 603-613.

Shephard, M.P. 1955. Resistance and tolerance of young speckled trout (*Salvelinus fontinalis*) to oxygen lack, with special reference to low oxygen acclimation. Journal of the Fisheries Research Board of Canada 12(3): 387-446.

Sprague, J.B. 1969. Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. Water Research 3: 793-821.

Stewart, N.E., D.L. Shumway, and P. Doudoroff. 1967. Influence of oxygen concentration on the growth of juvenile largemouth bass. Journal of the Fisheries Research Board of Canada 24: 475-494.

Subrahmanyam, C.B. 1980. Oxygen consumption of estuarine fish in relation to external oxygen tension. Comparative Biochemistry and Physiology 67A: 129-133.

US Census Bureau. 2003. North Carolina Quick Facts. quickfacts.census.gov/qfd/states/37000.html.

USDOC (U.S. Department of Commerce). 1996. Magnuson-Stevens Fishery Conservation and Management Act as amended through October 11, 1996. National Oceanic and Atmospheric Administration Technical Memorandum. NMFS-F/SPO-23.

Wagner, E.J., R.E. Arndt, and M. Brough. 2001. Comparative tolerance of four stocks of cutthroat trout to extremes in temperature, salinity, and hypoxia. Western North American Naturalist 61(4): 434-444.

Wannamaker, C.M. and J.A. Rice. 2000. Effects of hypoxia on movements and behavior of estuarine species. Journal of Experimental Marine Biology and Ecology 249(2): 145-163.

Whitworth, W.R. 1968. Effects of diurnal fluctuations of dissolved oxygen on growth of brook trout. Journal of the Fisheries Research Board of Canada 25(3): 579-584.

Wu, R.S.S., B.S. Zhou, D.J. Randall, N.Y.S. Woo, and P.K.S. Lam. 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environmental Science and Technology 37(6): 1137-1141.

Wu, R.S.S. 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Pollution Bulletin 45: 35-45.

YSI Model 52 Dissolved Oxygen Meter Operations Manual. 1998.

Appendix

SAS Version 8.2 Example Data Analyses

• Tank Effect Analysis Using Kaplan-Meier Method

```
data fish;
    input survtime status do wgt tank act_do temp species;
    datalines;
    0.00001     1     0.60   5.74   .     0.72   25     0
    0.00001     1     0.60   20.95  .     0.72   25     0
    0.00001     1     0.60   12.75  .     0.72   25     0
    .
    .
    .
    1060   0    1.20   7.15   11    1.28   30     1
    1060   0    1.20   6.85   11    1.28   30     1
    1060   0    1.20   7.00   11    1.28   30     1
    ;
proc sort;
    by species temp do;
    run;
proc lifetest plots=(s);
    by species temp do;
    time survtime*status(0);
    strata tank;
    run;
```

• Cox Regression Analysis of Menhaden

```
data men;
    input survtime status do wgt tank act_do temp species;
    time=survtime/60;
    datalines;
    0.00001     1     0.6    2.63    .     0.658333333   25     1
    0.00001     1     0.6    4.85    .     0.658333333   25     1
    0.00001     1     0.6    6.72    .     0.658333333   25     1
    .
    .
    .
    1440   0     1.2    8.33    2     1.233917526   30     1
    1440   0     1.2    6.51    2     1.233917526   30     1
    1440   0     1.2    10.28   2     1.233917526   30     1
    ;
data sub;
    input temp wgt act_do;
    datalines;
    25     4.40353     0
    25     4.40353     0.05
    25     4.40353     0.1
    .
    .
    .
    30     7.48247     1.4
    30     7.48247     1.45
    30     7.48247     1.5
    ;
proc phreg data=men;
    model time*status(0)=wgt act_do temp/ties=exact;
    run;
```

• Residual Analysis of Selected Model

```
proc phreg data=men;
    model time*status(0)=wgt act_do temp/ties=exact;
    output out=outp survival=surv;
    run;
data outp1;
    set outp;
    csres=-1*log(surv);
    run;
proc lifetest plot=(lls) notable;
    time csres*status(0);
    label csres='Cox-Snell Residuals';
    symbol c=black v=dot;
    run;
```

• Cumulative Hazard Function Plot of Selected Model

```
proc phreg data=men;
    model time*status(0)=wgt act_do temp/ties=exact;
    baseline out=base logsurv=ls;
    run;
data cum_haz;
    set base;
    ls=-ls;
    run;
proc gplot data=cum_haz;
    symbol1 value=none interpol=join;
    plot ls*time/haxis=0 to 24 by 4 vaxis=0 to 2.5 by 0.5 hminor=0
        vminor=0 ;
    title 'Cumulative Hazard Function';
    run;
proc print;
    var ls time;
    run;
```

• Predicting Specified Grid of Survival Function Estimates from Menhaden Model

```
proc phreg data=men;
    model time*status(0)=wgt act_do temp/ties=exact;
    baseline out=base covariates=sub survival=surv u=ucl l=lcl/nomean;
    run;
proc sort data=base;
    by temp;
    run;
data base2;
    set base;
    time=round (time,.0001);
    act_do=round (act_do,.01);
    surv=round (surv,.00001);
    ucl=round (ucl,.00001);
    lcl=round (lcl,.00001);
    run;
proc g3grid data=base2 out=outp;
    by temp;
    grid time*act_do=surv ucl lcl/axis1=0 to 24 by 1 axis2=0 to 1.5 by 0.05 join;
    run;
```

• Graphing Response Surface for Menhaden at 25°C

```
data men25;
    set outp;
    where temp=25;
    time=-time;
    run;
proc print data=men25;
    title 'Menhaden at 25C';
    run;
proc format;
    picture reverse
    low - <0 = '0009'
    0 = '0009';
    run;
proc g3d data=men25;
    title 'Effects of Low Dissolved Oxygen on Menhaden Mortality at 25C';
    title2 '(Mass = 4.40g)';
    format time reverse.;
    plot time*act_do=surv/grid ctop=black cbottom=gray rotate=45 zmax=1 zmin=0
        xticknum=7 yticknum=9 zticknum=6 caxis=black xytype=3;
    run;
```

• 12-Hour LC50 Value for Menhaden at 25°C

```
data men2512;
    input conc mort n @@;
    Concentration = conc; Observed = mort/n;
    datalines;
    0.6     148.5   149
    0.9     70.5    147
    1.2     0.5     149
    ;
proc probit log10;
    model mort/n=Concentration / d=normal inversecl;
    output out=new xbeta=xb p=Probability std=SE;
data new1;
    set new;
    Probit=probit(Probability)+5;
    run;
```

• LC50 Treatment Differences

```
data trt;
    input trt$ conc mort n @@;
    Concentration = conc; Observed = mort/n;
    datalines;
    1       0.6     148.5   149
    1       0.9     70.5    147
    1       1.2     0.5     149
    2       0.6     148.5   149
    2       0.9     126.5   165
    2       1.2     36.5    159
    3       0.6     149.5   150
    3       0.9     148.5   150
    3       1.2     26.5    146
    ;
proc probit order=data log10;
    class trt;
    model mort/n=trt Concentration / d=normal inversecl;
    title 'Probit Models for Treatment Differences';
    run;
```

• Kaplan-Meier Estimate Comparison between Acclimated and Non-Acclimated Fish

```
data km_acc;
    input time censor trt mass tank do acc species;
    cards;
    0.00001     1     0.60   2.63   .     0.66   0.00   1.00
    0.00001     1     0.60   4.85   .     0.66   0.00   1.00
    0.00001     1     0.60   6.72   .     0.66   0.00   1.00
    .
    .
    .
    180   0     0.60   3.61   11    0.58   1.00   0.00
    180   0     0.60   2.61   11    0.58   1.00   0.00
    180   0     0.60   2.08   11    0.58   1.00   0.00
    ;
proc lifetest plots=(s) outsurv=acca;
    time time*censor(0);
    strata acc species;
    title 'Acclimation';
    run;
proc print data=acca;
    run;
```