Evaluating hypoxia-inducible factor-1 α mRNA expression in a pelagic fish, Pacific herring
Clupea pallasii, as a biomarker for hypoxia exposure

Halley E. Froehlich^{1†*}, Steven B. Roberts¹, Timothy E. Essington¹

¹School of Aquatic and Fishery Sciences, University of Washington, Seattle, Washington 98105,
USA

*Present Address: National Center for Ecological Analysis and Synthesis, Santa Barbara,
California 93101, USA

[†]Corresponding author - Halley E. Froehlich, Ph.D.

froehlich@nceas.ucsb.edu

735 State Street, Suite 300, Santa Barbara, CA 93101

(206).375.4182

Steven B. Roberts: sr320@uw.edu

Timothy E. Essington: essing@uw.edu

Abstract

Hypoxia [dissolved oxygen (DO) < 2mg L⁻¹] is a major environmental perturbation for many aquatic ecosystems, particularly highly productive estuaries. Most research attention and understanding about the impacts of hypoxia on estuarine species has focused on the benthos, where hypoxia is most common. Although, the pelagic zone is also susceptible to the effects of hypoxia, the biological interactions and consequences are not as well understood in marine environments because documenting exposure or avoidance of hypoxia is often difficult. Physiological biomarkers may provide a way to gain more detailed spatiotemporal information regarding species' exposure to hypoxia. Here, we identified and tested a hypoxia-specific responsive gene, hypoxia-inducible factor-1 α (hif-1 α), to evaluate its potential as a biomarker for hypoxia exposure in Pacific Herring (*Clupea pallasii*), an abundant and widely distributed pelagic fish species. We conducted controlled laboratory experiments to establish the level of elevated gene expression (>1sd normoxic mean), exposure amplification ($\geq$ 2hrs), and reduction rate (ca. 24hrs) for hif-1 α . These experiments provided some evidence of a lethal hypoxic limit of Pacific herring (ca. 2 mg L⁻¹, $\geq$ 4hrs). We then used these findings to evaluate the spatiotemporal patterns of hif-1 α expression of Pacific herring in a seasonally hypoxia estuary, Hood Canal, Washington, U.S.A. Although gene expression did not parallel the local hypoxic conditions in the estuary, herring from the more severe hypoxic year (2013) had a higher probability of having elevated mRNA levels. These patterns indicate that hif-1 α mRNA levels may not be directly indicative of local DO levels, but rather provide insight into hypoxia exposure over broader scales. Moreover, this study demonstrates key differences and limitations of hepatic hif-1 α as a biomarker for a pelagic, highly mobile species versus more benthic organisms.

Keywords: hypoxia, hypoxia-inducible factor-1 α , biomarker, Pacific herring, Hood Canal, estuary

Introduction

While low oxygen events of hypoxia and anoxia are becoming more frequent, severe, and expansive in marine ecosystems, in offshore waters such conditions are commonly intermittent and reside in deeper areas (Diaz and Rosenberg, 2008; Stramma et al., 2010). Because hypoxia [dissolved oxygen (DO) < 2 mg L⁻¹] is more common in the deeper reaches of these aquatic environments, most investigation into the ecological impacts of hypoxia has focused on demersal and benthic species (e.g., (Diaz and Rosenberg, 2008; Levin et al., 2009; Riedel et al., 2014). Yet, hypoxia also extends vertically into the water column, but the impacts on marine pelagic organisms are not as well understood (Ekau et al., 2010; Pollock et al., 2007; Zhang et al., 2009). Given that the pelagic zone contains some of the most ecologically and commercially important species (e.g., forage fish), a deeper understanding of the implications of hypoxia is critical for improved conservation and management of these threatened ecosystems.

Indirect and/or sub-lethal effects of hypoxia are the most common biological consequences for marine pelagic organisms and require a multitude of approaches, such as biomarkers, to measure such responses. Numerous studies have detected avoidance responses to hypoxia, resulting in habitat compression and/or reduced access to prey (i.e., zooplankton) (Longhurst, 1967; Ludsins et al., 2009; Parker-Stetter and Horne, 2009; Prince and Goodyear, 2006; Vanderploeg et al., 2009b; Zhang et al., 2009). However, depending on the system, species, and severity of the hypoxic conditions, pelagic fish (e.g., Pacific herring) have also been documented inhabiting low oxygen habitats, presumably exploiting prey within the hypoxic layer (Parker-Stetter et al., 2009; J. J. Roberts et al., 2012; Taylor et al., 2007; Zhang et al., 2009). While these movements into low-DO water may provide foraging opportunities, exposure to reduced oxygen levels can negatively impact critical demographic rates, including growth, reproduction, and overall survival (e.g., disease, predator-avoidance) (Domenici et al., 2007, 2002; Ekau et al., 2010; Gilly et al., 2013; Riedel et al., 2014; Pollock et al., 2007; Wu, 2002; Zhang et al., 2009). However, the extent and duration of exposure is often difficult to directly observe via standard monitoring frameworks (e.g. acoustics, capture sampling) without dependable biological markers (biomarkers) (Kodama et al., 2012b) – particularly if the fish are making brief forays into the low DO conditions.

In general, biomarkers can capture measurable changes in a biological response (e.g., gene expression) which help to elucidate levels of exposure to a particular stressor that can then be

used to infer spatial and temporal patterns of a species relative to the environmental landscape. For instance, the use of species-specific biomarkers is common for monitoring pollutants in marine environments (e.g., Wang et al., 2008). One major advantage of a biomarker is the ability to detect sub-lethal impacts at low levels of stressor intensity. Moreover, implications about consequences of sub-lethal exposure can be made more robustly because the results gleaned from biomarkers can provide detailed physiological information about sentinel species. A useful biomarker needs a sufficient level of specificity to the stressor of interest, is easy to assay, and relatively unaffected by sampling procedures (Zhang et al., 2012). In addition, biomarker selected species should have a broad geographic range, be broadly distributed among habitats, and be sufficiently common and accessible to make monitoring less costly. That said, the use of biomarkers in monitoring is not a substitute for water quality measurements or population observations, but is an integrative technique to assist in evaluating exposure to stressors.

Animals can often remain in low oxygen by increasing specific oxygen-dependent regulatory transcriptional proteins known as hypoxia-inducible factors (HIFs) (Bracken et al., 2003). HIFs (HIF-1 α , HIF-2 α , and HIF-3 α) can mediate oxygen efficiency and the suppression of metabolic rates, shifting energy production and function towards optimizing oxygen use and reserves – the most ubiquitous isoform being HIF-1 α (Bracken et al., 2003; Nikinmaa and Rees, 2005). Under normoxic conditions hif-1 α mRNA and HIF-1 α protein are continuously produced at low levels, but the protein sub-unit is almost just as rapidly degraded (Bruick, 2003). HIF-1 α stabilizes when oxygen is limited, resulting in a cascade of gene regulation (200+ genes) and associated biological effects (Bruick, 2003; Wenger, 2006). While regulation of the post-translational form of HIF-1 α (i.e., protein) is well understood, the mechanism governing hif-1 α gene expression (i.e., mRNA) is less clear, particularly for fish (Chen et al., 2012). However, laboratory studies of aquatic organisms have shown evidence of increased hif-1 α gene expression in response to low oxygen levels (Bracken et al., 2003; Kodama et al., 2012a, 2012b; Shen et al., 2010; Thomas et al., 2007; Thomas and Rahman, 2009). As a result, mRNA of hif-1 α may prove an informative tool for evaluating biologically significant hypoxia exposure. Regardless of the form of expression, the level depends on the organism and tissue type.

Field studies on benthic marine organisms have showed that hif-1 α mRNA levels become significantly elevated in tissues collected from hypoxic sites compared to specimens from normoxic sites (Kodama et al., 2012a; Thomas and Rahman, 2009). However, the field

component of these studies only spanned short time periods (days) during a single hypoxic event and exclusively focused on bottom-oriented species. Further, a unifying DO threshold and temporal pattern of hif-1 α gene expression has not been established (Thomas and Rahman, 2009). The biological signature may dampen (i.e., acclimation) (Law et al., 2006) or persist (i.e., continual expression to buffer deleterious hypoxic effects) (Thomas et al., 2007) over time depending on the species. Thus, it is uncertain how, or even if, hif-1 α mRNA levels can be used to reliably detect exposure or hypoxia avoidance in many species.

In Washington State, USA, several basins within the large marine ecosystem of Puget Sound experience seasonally hypoxic episodes. Composing the western arm of Puget Sound, Hood Canal (110 km) typically exhibits hypoxic periods during the summer months when stratification restricts mixing throughout the water column (Newton et al., 2007). The factors driving the hypoxic conditions in Hood Canal include the bathymetry that reduces tidal currents and hydrological exchange rates from north to south, coupled with the oceanographic input of low oxygenated, upwelled waters (Cope and Roberts, 2013; Newton et al., 1995). As a result, bottom water DO (mg L⁻¹) levels in the southern regions decline throughout summer, while simultaneously hypoxia expands vertically into the pelagic, shallow reaches (20-30m) of the estuary (Newton et al., 1995). Although hypoxia has been reported in Hood Canal since the 1950s, further deterioration of the oxygen conditions due to anthropogenic climate change and nutrient loading is a major concern (Fagergren et al., 2004; Newton et al., 1995).

The aim of the present study was twofold: (1) develop a molecular assay to assess the magnitude and temporal changes in hif-1 α expression following acute hypoxia exposure; and (2) describe temporal and spatial patterns of acute hypoxia exposure using the assay for Pacific herring (*Clupea pallasii*), one of the most abundant pelagic fishes in coastal and estuarine ecosystems in the Northeast Pacific Ocean. Specifically, we used a hepatic transcriptome of Pacific herring to develop a qPCR assay for hif-1 α . Liver tissue was selected because of the rapid cellular turnover (potentially most responsive to acute exposure), elevated hif-1 α detection in other species (Chen et al., 2012; Rimoldi et al., 2012; Terova et al., 2008), and the sequenced transcriptome (S. B. Roberts et al., 2012). We then tested the threshold and time-course of gene expression through laboratory, oxygen-treatment experiments and used those results to interpret expression patterns of herring liver tissue collected from Hood Canal. In the laboratory, we hypothesized that (1) under a threshold experiment, gene expression would be higher for hypoxia

treated fish relative to controls and (2) under a time-course experiment, hypoxia induced gene expression would exhibit a dose-like response over time, and rapidly return to baseline levels once returned to normoxic conditions. In the field, we hypothesized hif-1 α mRNA expression would track the spatiotemporal patterns of Hood Canal by (1) elevating in the south compared to the north as hypoxia increased over the summer months (June – October) and (2) would increase overall under more severe hypoxic conditions. Although not a primary focus of this study, we also evaluated the effect of size for both the lab and field components, as previous work has suggested a relationship between hypoxia tolerance and size, with smaller individuals tending to be more susceptible to the impacts of hypoxia (Nilsson and Ostlund-Nilsson, 2008).

Material and Methods

Laboratory Experiments

Laboratory experiments were conducted in 2013 at the U.S. Geological Survey (USGS) Marrowstone Marine Field Station (MMFS), Nordland, Washington, U.S.A. All laboratory experiments used specific pathogen free (SPF) Pacific herring [mean fork-length (FL) $\pm$ SD = 129.0 ± 9.0 mm] reared at MMFS (stock no. SPF 12-2). The source location of the Pacific herring was Cherry Point, Washington. Prior to all experiments, fish were transferred to experimental tanks and allowed to acclimate for approximately 24 hours. Temperature was held relatively constant for all experiments (mean temperature $\pm$ SD = $12.8 \pm 0.5^{\circ}\text{C}$).

To determine the oxygen level that induces elevated hif-1 α gene expression, we conducted a *threshold experiment* with two treatment groups and one control. Each treatment group consisted of six Pacific herring. Hypoxic treatment groups were exposed to the lowest DO conditions (mean DO $\pm$ SD = 2.63 ± 0.40 mg L⁻¹), the second treatment to moderate levels (4.25 ± 0.31 mg L⁻¹), and the control groups kept at normal DO conditions (6.84 ± 0.08 mg L⁻¹); the range of DO levels corresponded to possible oxygen levels experienced by Pacific herring in their natural environment. In the flow-through system, DO levels were slowly reduced over a 2 hour acclimation period by bubbling nitrogen (N₂) gas into a separate reservoir (1600L), which flowed (ca. 4.5 L min⁻¹) into the treatment tanks (760L). All tanks were fitted with opaque, plastic covers placed at the water-air interface to maintain constant oxygen levels. In addition, a circulating-pump was placed inside the reservoir to homogenize the oxygen conditions throughout the container. DO and temperature conditions were continuously monitored and N₂

gas controlled through a data acquisition automated system that we developed using LabVIEW 2012 and Vernier DO and temperature probes. After 16 hours of exposure, individuals were euthanized (MS-222) and liver tissue extracted, placed in RNA*later*® (Life Technologies Corporation, Carlsbad, CA), and stored at -20°C for further processing. The experiment was replicated a total of four times.

To test for a dose-like response of *hif-1α* mRNA expression to hypoxia, we ran a *time-course experiment* where Pacific herring were exposed to hypoxia for several hours and then returned to normoxic conditions, while being sampled throughout. Similar to the threshold experiment, DO levels were slowly reduced (2hr) to the lowest oxygen level (mean DO $\pm$ SD = 2.31 ± 0.41 mg L⁻¹). Beginning with 32 herring in a single treatment tank, four herring were consecutively sampled at a geometric time interval from 0 to 16 hours after the initiation of hypoxic treatment; the same sampling procedures from the threshold experiment were applied. After 16 hours of exposure, DO concentrations in the treatment tanks were returned to normoxic levels (6.60 ± 0.28 mg L⁻¹) by increasing flow and aerating the tanks. At 12 and 24 hours after returning to normoxic conditions, four herring were then sampled from each tank as described above. Again, the time-course treatment was replicated four times and liver tissue extraction and preservation were the same. Regarding both experiments, any herring displaying loss of equilibrium were documented and immediately euthanized.

Field Sampling

Pacific herring were captured monthly from June – October in 2012 and 2013 at hypoxia prone sites using mid-water trawls. The site-based sampling design included two sites in the southern region that experience the strongest hypoxia (sites A and B; DO < 2 mg L⁻¹), one site farther north in the main channel with light-to-moderate hypoxia (site C; DO > 2 mg L⁻¹), and the reference, normoxic site in the far north (site D; DO > 3; Fig. 1); pacific herring at site D were only captured in October – typically the most low oxygen time period in Hood Canal. Sites B, C, and D have depths ranging from ca. 100 to 200m, while the southern-most site (A) has a maximum depth of 70m. We expected differences in the timing, intensity, and vertical intensity of hypoxia among these sites, so measuring all four throughout two seasons allowed us to separate phenological effects unrelated to hypoxia from those related to hypoxia. In addition, all field observations were accompanied by synoptic monitoring of oxygen and temperature profiles

using a Sea-Bird (SBE) 9plus SEACAT profiler, fitted with dissolved oxygen and pH auxiliary sensors.

We collected five Pacific herring at each site during each month (except site D) over the two-year sampling period. Sampled fish were relatively similar in size, with mean FL =173.4 mm and a coefficient of variation of less than 10%. No herring were captured during the 2013 July collection period at site C. Acoustic surveys determined areas and depths to be sampled with a Marinovich mid-water trawl. Fish were emptied from the trawl into a well oxygenated holding tank, and five herring were haphazardly collected immediately, and euthanized using an overdose of MS-222. Herring liver tissue was extracted immediately after death, placed in RNAlater®, and stored at -20°C for later genetic processing.

RNA isolation and cDNA synthesis

All liver tissue from laboratory experiments and field sampling was processed and cDNA synthesized. First, total RNA was extracted from herring tissue using TriReagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's protocol. To eliminate any remnant genomic DNA, the RNA samples were treated with Ambion® DNA-free™ DNase (Life Technologies Corporation, Carlsbad, CA). Purified RNA was then quantified by optical density at 260nm using a UV spectrophotometer (NanoDrop ND-1000). Quantitative PCR (qPCR) was performed on treated RNA to ensure absence of DNA carryover. Finally, cDNA was synthesized from the RNA (1µg) using a reverse transcription (RT) kit (Promega Corporation, Madison, WI). First 17.75µl of RNA and 0.5µl of oligo-dT primers were incubated at 70°C for 5min, then immediately placed on ice. For a 25µl reaction, 6.75µl of master mix containing 5µl of M-MLV RT Buffer, 1.25µl of 2.5mM dNTP, and 0.5µl M-MLV reverse transcriptase was added to the primed RNA. The solution was then incubated at 42°C for 1hr, and heat inactivated at 95°C for 3min. The integrity of the cDNA was then checked using the UV spectrophotometer and ultimately stored at -20°C.

Assay development

In order to identify the target sequence a hepatic transcriptome (S. B. Roberts et al., 2012) was annotated using the GenBank database and National Center for Biotechnology Information (NCBI)-Basic Local Alignment Search Tool (BLAST) algorithm (McGinnis and

Madden, 2004; Morgulis et al., 2008; Zhang et al., 2000). Use of the BLASTx algorithm identified the sequence as hif-1 and its associated complexes (i.e., PAS domain and hif-1 α CTAD). The BLASTn program was then implemented to construct a dendrogram (tree) based on alignments to visualize and compare species with the most similar nucleotide sequences (homologs) to the herring hif-1 α sequence. Primers were then developed with NCBI Primer-BLAST (forward 5'- GTTGAGCAGCTTCCTCATGC-3'; reverse 5'- GGAGTCGGAGGTGTTCTACG-3'; accession no. **PR032251225**). The level of pairwise congruence of the herring sequence and primer region was then cross-compared to three sequenced isoforms (hif-1 α , -2 α , and -3 α)¹ from Zebrafish (*Danio rerio*) and Chinese sucker (*Myxocyprinus asiaticus*) to corroborate isoform specificity.

To confirm primer amplification size we ran conventional polymerase chain reaction (cPCR) in a 1.3% agarose gel. In a 25ul reaction, cPCR was performed using 12.5ul 2x Apex Red (Promega Corporation, Madison, WI), 0.5ul of forward and reverse primer (10uM) each, 9.5ul of ultra-pure water, and 2ul of template cDNA. Cycling parameters for cPCR were as follows: (1) 95°C for 10min (2) 39 cycles of 95°C for 15s, (60-65)°C for 15s, then 72°C for 30s, and (3) 72°C for 8min. Products were compared using an annealing temperature gradient spanning 60-65°C. Similar methods were used to develop the assay for our normalizing gene, elongation factor-1 α (EF-1 α), a common housekeeping gene (forward 5'- AGAGCAATGTCAATGGTGAT-3'; reverse 5'-TCTCATCTACAAATGCGGAG-3'; accession no. **PR032251224**). Although we were restricted to one normalizing gene, EF-1 α did appear stable across treatments.

Quantitative PCR

For amplification and measurement gene expression, qPCR was performed in a 20ul reaction mixture using 10ul of 2x SSoFastTM EvaGreen (Bio-Rad Laboratories, Inc., Hercules, CA), 0.5ul of forward and reverse primer (10uM) each, 8ul of ultra-pure water, and 1ul of cDNA template. PCR cycles were as follows: (1) 95°C for 30s, (2) 95°C for 5s, (3) 60°C for 5s, (4) plate read, (4) return to step two 39 more times, and (6) melt curve from 65°C to 95°C, at 0.5°C for 3s (+plate read). Amplification and melt curves were evaluated and relative fluorescence

¹ *Danio rerio* hif-1 α (**AY326951**), hif-2 α (**DQ375242**), hif-3 α (**AY330295**); *Myxocyprinus asiaticus* hif-1 α (**HQ432957**), hif-2 α (**HQ432955**), hif-3 α (**HQ432956**).

units (RFU) quantified using PCR Miner software (Zhao & Fernald 2005). Average gene expression was then calculated using cycle threshold (CT) and average efficiency (AE) from the equation, mRNA expression = $(1+AE)^{-CT}$ (Zhao & Fernald 2005). The hif gene expression was normalized by dividing by the corresponding EF-1 α values. All samples were run in duplicate.

Analysis

The laboratory threshold and time-course data were analyzed separately using a two-way analysis of variance (ANOVA), with a post-hoc Tukey's test. We specifically evaluated the relationship of mean gene expression levels of hif-1 α to treatment (level of DO or length of exposure) and size of the fish [fork-length (mm)]. We used a level of 0.05 to determine significant differences among treatments and/or time periods. Data were also approximately normal distributed with constant variance.

The field data were analyzed by separately modeling the frequency of elevated gene expression in relation to key spatial, temporal, and biological variables. Given hif-1 α mRNA is continuously produced and the complexity of herring behavior and environmental heterogeneity, evaluating occurrence (number of fish) of elevated expression rather than the mean expression provided an improved approach to capture patterns of hif-1 α elevation. Inferred by the laboratory results, we tested the probability of significant increases in mRNA expression – more than 1 standard deviation (sd) above mean reference (site D) expression levels – relative to several potential predictor variables. We assessed the frequency of elevated expression by using a generalized linear model, a binomial probability density function, and a logit link function to model the proportion of herring exhibiting high levels of hif-1 α (Gelman and Hill, 2010). The binomial model evaluated the relationship between the probability of elevated hif-1 α mRNA expression relative to sampling year (2012, 2013) or month (June - October), sampling site (A, B, C), fork-length of each fish (mm), and the mean trawl depth (m) at which the herring were captured. Monthly effects were analyzed separately for each year due to the strong interannual differences. We considered statistically significant covariates with p values less than 0.05. All statistics were performed in R (R Core Team, 2013). Data and R script are publically available (Froehlich et al., 2014).

Results

Assay Development

The Pacific herring sequence (858 bp; Contig8790) (S. B. Roberts et al., 2012) and associated primer pairs exhibited a high level of agreement with one isoform, hif-1 α . The putative herring hif-1 α sequence showed identities $\geq 75\%$ and query coverage $\geq 73\%$ with zebrafish, Chinese sucker, common carp (*Cyprinus carpio*), Crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*), asp (*Aspius aspius*) stone moroko (*Pseudorasbora parva*), and Mexican tetra (*Astyanax mexicanus*). In addition, only hif-1 α of the zebrafish and Chinese sucker displayed high levels of identity with the herring sequence, while no significant similarities were found with the other isoforms (hif-2 α & -3 α). Relative to the primer region of the herring sequence, the zebrafish and Chinese sucker shared 80% and 84% identical base pairs, respectively. Lastly, cPCR confirmed the expected product length (129 bp) predicted from the primer pairs.

Threshold experiment

While the qPCR results showed detectable levels of expression of hepatic hif-1 α in all treatments, gene expression was significantly higher ($F_{2, 67} = 3.29$, $p = 0.0435$) for fish exposed to the lowest oxygen conditions (2.63 ± 0.40 mg L⁻¹). Average hif-1 α mRNA expression of Pacific herring after 16hrs of hypoxia exposure was approximately two times higher than fish under moderate or control conditions (Fig. 2). In addition, mean expression of the moderate and control groups were not significantly different. Similarly, there was no significant effect of size ($F_{1, 67} = 0.003$, $p = 0.956$). We note that six fish from the hypoxia treatment groups lost equilibrium and were euthanized an average of 8.2hrs. We considered such events mortalities. Conversely, no moderate or control fish specimens perished prior to the end of any replicate trial.

Time-course experiment

Post acclimation, Pacific herring liver tissue showed a clear temporal increase in mean hif-1 α mRNA expression over the majority of hypoxic exposure, and a rapid decline after conditions were returned to normoxic levels. Relative to average control expression, hif-1 α gene expression did not significantly increase until 2hrs, and peaked at 8hrs, into the experiment ($F_{9, 152} = 2.31$, $p = 0.0185$; Fig. 3). Although the 16hr hif-1 α gene expression levels were similar

to levels seen in the threshold hypoxia treatment (ca. 2x the control), the time-course experiment revealed a significant decrease in overall expression. Once the Pacific herring were returned to normal oxygen conditions (i.e., recovery period), hif-1 α gene expression continued to decline (Fig. 3). Given the low level of expression of the control and potential influence of the 2hr acclimation, mRNA levels greater than one standard deviation from the control mean appeared to be significantly above the normal expression variability; we thus used this as the defining measure for identifying significantly elevated expression. In addition, hif-1 α expression generally returned to baseline levels 24hrs after exposure (Fig. 3). Again, there was no significant difference ($F_{1, 152} = 2.58, p = 0.11$) in mean expression relative to size. Similar to the threshold experiment, five fish suffered mortality an average of 6.0hrs from initiation of the experiment.

Field collection

Monthly dissolved oxygen profiles showed discernable differences in hypoxic distribution over the sampling sites and between the two study periods. Site A, the most shallow and hypoxia prone location, displayed higher oxygen levels at the beginning of the study in 2012 compared to 2013 (Fig. 4). However, during both years oxygen conditions declined throughout the summer, becoming most depleted (ca. 2 mg L⁻¹) in October and August of 2012 and 2013, respectively. Site B, the deeper southern site, also had higher oxygen levels in 2012, but in both years these levels declined seasonally, reaching to hypoxic levels in 2013 (Fig. 4). Site C, the deepest of the sampling sites, displayed the most similar pattern across both years, with higher levels of DO during both sampling periods that slowly declined and expanded over time (Fig. 4). However, similar to all other hypoxia impacted sites, 2013 conditions were more severe than in 2012. It is important to note that the deepest portion of site C (160 – 200m) was not measured (Fig. 4), but can experience severe hypoxia year around. Site D, the shallow northern reference, maintained better water quality over both study seasons compared to all other sites, consistently staying near or above 4mg L⁻¹. All Herring were captured in DO conditions greater than 2mg L⁻¹.

The probability of elevated hif-1 α mRNA expression was significantly different between site and year (Table 1). Despite expectation, the probability of elevated hif expression was independent of sampling month, thus we focus on the year effect model. The probability of elevated hepatic hif-1 α gene expression was greatest in the northern site (C) and lowest in the

most southern site (A; Fig. 5) –counter to the hypoxia gradient. In fact, the majority of herring captured in the lowest oxygen conditions (2-3 mg L⁻¹), typically in site A, did not exhibit elevated levels (Fig. 4 & 5). However, consistent with more severe hypoxic conditions inducing a response, the model coefficients showed, on average, herring in 2013 had three times higher probability of exhibiting increased hif-1 α expression compared to fish captured in 2012 (Fig. 5). Akin to the laboratory results, size was not a statistically significant variable.

Discussion

Our findings provide, to our knowledge, the first evaluation of hepatic hif-1 α as a biomarker for hypoxia exposure in a forage fish species, Pacific herring. In the laboratory, we were able to establish the level of elevated gene response (>1sd of normoxic levels), exposure amplification ($\geq$ 2hrs), and reduction rates (ca. 24hrs) of hif-1 α mRNA expression. We also uncovered evidence of a hypoxic lethal-limit for the herring species. However, in field the spatial gene expression did not track the local patterns of hypoxic conditions, most likely due to the highly mobile nature of the species. Yet, herring from the more severe hypoxic year (2013) had a higher probability of evaluated mRNA levels, suggesting the fish will or have to enter lower oxygen conditions when water quality is more deteriorated.

In the laboratory, we detected increased levels of hif-1 α mRNA resulting from acute hypoxia exposure and verified reduction in expression upon returning to normoxic conditions. The hypoxia-experiments revealed a clear pattern of increased hepatic hif-1 α expression under oxygen conditions around 2mg L⁻¹, similar to studies on Eurasian perch (*Perca fluviatilis*), sea bass (*Dicentrarchus labrax*) and Chinese sucker (Chen et al., 2012; Rimoldi et al., 2012; Terova et al., 2008). In addition, mRNA expression returning to baseline levels within 24hr is also consistent across multiple studies and species, tolerant and sensitive alike (Kodama et al., 2012b; Rahman and Thomas, 2007; Rimoldi et al., 2012; Terova et al., 2008). Although there is a notable range of ‘hypoxic’ levels (0.4-4.3 mg L⁻¹), exposure times (1-24hrs), and sampling intervals between different studies due to species-specific variability, the general pattern appears fairly ubiquitous. The 24hr reduction in hif-1 α expression is particularly informative, providing a key insight into the timing of exposure in a field setting that cannot be gleaned from other

common sampling methods alone (e.g., trawling). Based on these findings, hepatic hif-1 α gene expression could prove to be a viable and robust biomarker across numerous species.

Our experiments also indicate the lethal-threshold of Pacific herring to hypoxia. For the forage fish species, 4 to 8hrs of exposure to low DO conditions (2.31 ± 0.41 mg L⁻¹) resulted in the highest hif-1 α expression levels. However, that similar time window also had the potential for mortality induced effects (i.e., loss of equilibrium), suggesting a lethal limit of hypoxia exposure. Moreover, expression of hif-1 α plummeted after 16hrs, most likely due to suppression of overall transcription and translation and not acclimation. Typically, when an organism becomes stressed and homeostatic conditions cannot be achieved, cellular energy is shunted towards cardiac and neural function and down-regulated in other, non-essential tissues (Gorr et al., 2010). However, given the highly mobile nature of pelagic fish, it seems unlikely Pacific herring in the wild would be exposed to such conditions. Nonetheless, the time course laboratory experiments allowed us to reveal the full scope of the hif-1 α mRNA response, establishing the baseline variability, and how that relates to hypoxia lethality. While other studies report that hif peaks and troughs with prolonged hypoxia exposure (Law et al., 2006; Rimoldi et al., 2012), the fine scale association of lethality presented in this study is unique.

We found evidence that more severe hypoxic conditions on an interannual scale increased the probability of elevated genetic expression of hif-1 α , and thus exposure to hypoxia. Specifically, Pacific herring sampled during the more intense 2013 hypoxia event had a greater probability of elevated hif-1 α expression compared to 2012. These results suggest that the herring are more willing and/or obliged to enter hypoxia when low oxygen conditions are more severe. Similar to responses reported for more benthic-oriented species, exposure is most likely context-dependent to the level and extent of the poor water quality conditions (Eby and Crowder, 2002). As result, if DO levels continue to decline due to anthropogenic influences of climate change and nutrient loading (Newton et al., 2007), Pacific herring, and potentially other estuarine species in the system, could be at risk for increased exposure and the associated biological ramifications (e.g., increased disease, depressed fecundity, reduced growth) (Ekau et al., 2010; Wu, 2002).

The hif-1 α gene expression did not however mimic the local conditions of hypoxia, suggesting exposure, adaptation and/or avoidance of the highly mobile species at a broader spatial scale. Pacific herring showed the most significant elevated expression of hif-1 α in the least hypoxia impacted site (C), suggesting herring may be making brief forays into the deepest,

more hypoxic reaches of the estuary for foraging or predator avoidance (Parker-Stetter et al., 2009; J. J. Roberts et al., 2012; Taylor et al., 2007; Zhang et al., 2009). Indeed, Pacific herring can move hundreds of kilometers in a matter of days (Haegele and Schweigert, 1985) and our hypoxic sites were only 12-18km apart. In addition, herring captured in the lower DO areas (site A and B) with lower potential of elevated hif-1 α may be adapted, acclimated, or avoiding the most severe hypoxia all together. While acclimatization could be occurring, similar low oxygen levels resulted in mortalities and lower levels of expression after 16hrs of exposure in the laboratory. However, as the laboratory herring were not from the Hood Canal basin there is a higher potential for the field herring to be better adapted to hypoxic conditions. In fact, species that experience hypoxia more frequently may be more tolerant compared to naïve organisms (Sagasti et al., 2001), especially if the tolerance-trait is hereditary. Alternatively, the lack of elevated expression may suggest the Pacific herring were more restricted to higher oxygen levels in the water column (Horppila et al., 2000; Ludsin et al., 2009; Parker-Stetter and Horne, 2009; Taylor et al., 2007; Vanderploeg et al., 2009b). Lending more credence to an avoidance response, herring were never captured in conditions less than 2mg L⁻¹. Regardless, the counterintuitive results highlight the difference between lab and field conditions and the importance of sampling over multiple sites and times.

While it is unclear the exact combination of circumstances leading to the hif-1 α gene expression patterns in the field, it is certain that pelagic species behavior and responses are more variable than their demersal counterparts. The ability of forage fish to quickly move horizontally and vertically in the water column appears to result in different exposure and oxygen demand patterns than those reported for more benthic-oriented organisms (Vanderploeg et al., 2009a, 2009b). Species such as Atlantic croaker (Thomas and Rahman, 2009) and mantis shrimp (Kodama et al., 2012b) consistently show high expression in hypoxic field sites, which we did not find for Pacific herring. However, more similar to our findings, Thomas and Rahman (2009) reported elevated levels of hif-1 α in a central normoxic location of their field study, which they attributed to movement of the fish from the adjacent hypoxic waters to a more oxygenated area. Although the increased flexibility of movement within the water column results in more a complex expression pattern, it does appear lower DO levels are affecting herring hif-1 α expression.

Conclusions

This ecophysiological research offers a unique approach to understanding the influence of hypoxia on a pelagic marine species. We found acute exposure (2-8hrs) to DO between 2-3mg L⁻¹ significantly increased the gene expression of hif-1 α , but simultaneously caused detrimental effects as time progressed. In addition, the 24hr decrease of hif-1 α was consistent with numerous other studies. In the field, while probability of expression increased during the more severe low DO event, the spatial pattern was counter to the spatial hypoxic gradient spanning south to north. These results show that while hepatic hif-1 α does not translate directly to the local hypoxic conditions, it did capture broader spatial and temporal signals of hypoxia's influence. Although the use of hif-1 α gene expression as a biomarker for hypoxia exposure provided quantitative measures and means to better infer potential biological implications, the complex behavior and energy demands of Pacific herring made field interpretation less clear than those of more benthic-oriented species. As a result, more in-depth evaluation of movement patterns and connectivity of Pacific herring in Hood Canal, and other basins of Puget Sound, is needed to fully understand the responses of this important forage fish species to hypoxia and other environmental stressors.

Acknowledgements

Thanks to Sam White for his guidance and answering of copious amounts of genetic related questions. Gratitude and thanks to Paul Hershberger, Jacob Gregg, Lucas Hart, Brad Tyler, and Naomi Lewandowski for the fish and experimental space at the USGS MMFS. We also thank Eric R. Nelson for his expertise in computer engineering and LabVIEW implementation. Additional thanks to Pamela Moriarty for collecting liver tissue when others could not. The manuscript was improved by comments from P. Sean McDonald and Charles 'Si' Simenstad. In all, this research could not have been accomplished without the help and kindness of all of these amazing individuals. This research was funded by the National Science Foundation (OCE-1154648), the Northwest Scientific Association, and the University of Washington, School of Aquatic and Fishery Sciences. Funding of H.E. Froehlich was also provided by NSF through the Graduate Research Fellowship Program (DGE-0718124; DGE-1256082). All research was conducted in accordance with institutional, state, national, and international guidelines regarding the use of animals in research (IACUC Protocol No. 4062-09).

Table 1. Analysis of deviance table for model predicting probability of elevated (>1 sd from reference mean) hif-1 α mRNA expression. Depicted are the main effects: sampling year (2012, 2013), sampling site (A, B, C), size of fish (mm), and mean trawl depth (m); terms added sequentially (first to last). P-values less than 0.05 were considered statistically significant.

	df	Deviance	Residual df	Residual Deviance	p-value ($>\chi^2$)
Intercept			136	141.43	
Year	1	4.8611	135	136.57	0.02747
Site	2	6.4596	133	130.11	0.03957
Size	1	1.5805	132	128.53	0.20869
Trawl Depth	1	0.07570	131	127.77	0.38427

Figure 1. Study sites in Hood Canal, Washington. From June to September dissolved oxygen (DO) declines in the estuary, but southern sites (A, B) experience more rapid and intense low oxygen levels. By late summer, both southern sites have hypoxic and/or anoxic layers. Site C water quality also declines, but typically gets only moderately hypoxic. Site D, the reference sampling site, tends to have the most consistent and highest ($\geq 4 \text{ mg L}^{-1}$) DO conditions.

Figure 2. Relative mean ($\pm$ S.E.) normalized hif-1 α gene expression of Pacific herring liver tissue from threshold experiments. Pacific herring were exposed to 16hrs of control (mean dissolved oxygen $\pm$ SD = 6.84 ± 0.08 mg L⁻¹), moderate (4.25 ± 0.31 mg L⁻¹), and hypoxic (2.63 ± 0.40 mg L⁻¹) conditions. Expression levels are relative to the control. Differences in mean mRNA levels between the control treatment and other treatments were tested by using a two-way ANOVA, * $p < 0.05$.

Figure 3. Relative mean ($\pm$ S.E.) normalized hif-1 α gene expression of Pacific herring liver tissue from the time-course experiment. Herring were sampled at a geometric time interval from 0 to 16 hours of hypoxia (mean dissolved oxygen $\pm$ S.D. = 2.31 ± 0.41 mg L⁻¹) exposure (*dark gray*). After 16hrs, oxygen conditions were returned to normal (6.60 ± 0.28 mg L⁻¹) and samples were taken 12hr and 24hrs post-hypoxia treatment (*light gray*). ‘Control’, depicted in *white*, corresponds to mean expression level at 16hr. Expression levels are show relative to the ‘control’. Differences in mean mRNA levels between the normoxic treatment and other time-steps were tested by using a two-way ANOVA, * $p < 0.05$.

Figure 4. 2012 (*top*) and 2013(*bottom*) interpolated dissolved oxygen (mg L^{-1}) over all sample periods (June-October) and sites (A, B, C, and D). Each DO graph represents a sampling site depicting the contour (*black lines*) and gradient (*color*) of DO as depth and time increase. The *black Xs* represent the mean trawl depth (m). The *gray* color represents the unsampled and/or maximum depths. Specifically, site C maximum depth is 200m, but was not measured. Interpolation was performed in R using the *interp* function, which applies a bilinear interpolation to the data.

Figure 5. Number of fish exhibiting elevated *hif-1 α* mRNA expression (i.e., >1sd mean norm) across the three hypoxia prone sampling sites (A, B, and C) and times (June-October). Site D (October; not pictured) was the reference site.

References

- Bracken, C.P., Whitelaw, M.L., Peet, D.J., 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. *Cell. Mol. Life Sci.* 60, 1376–1393. doi:10.1007/s00018-003-2370-y
- Bruick, R.K., 2003. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes Dev.* 17, 2614–2623. doi:10.1101/gad.1145503
- Chen, N., Chen, L.P., Zhang, J., Chen, C., Wei, X.L., Gul, Y., Wang, W.M., Wang, H.L., 2012. Molecular characterization and expression analysis of three hypoxia-inducible factor alpha subunits, HIF-1 alpha/2 alpha/3 alpha of the hypoxia-sensitive freshwater species, Chinese sucker. *Gene* 498, 81–90. doi:10.1016/j.gene.2011.12.058
- Cope, B., Roberts, M., 2013. Review and Synthesis of Available Information to Estimate Human Impacts to Dissolved Oxygen in Hood Canal. Washington State Department of Ecology & Environmental Protection Agency, Olympia, WA.
- Diaz, R.J., Rosenberg, R., 2008. Spreading Dead Zones and Consequences for Marine Ecosystems. *Science* 321, 926–929. doi:10.1126/science.1156401
- Domenici, P., Ferrari, R.S., Steffensen, J.F., Batty, R.S., 2002. The effect of progressive hypoxia on school structure and dynamics in Atlantic herring *Clupea harengus*. *Proc. R. Soc. Lond. Ser. B-Biol. Sci.* 269, 2103–2111. doi:10.1098/rspb.2002.2107
- Domenici, P., Lefrancois, C., Shingles, A., 2007. Hypoxia and the antipredator behaviours of fishes. *Philos. Trans. R. Soc. B-Biol. Sci.* 362, 2105–2121. doi:10.1098/rstb.2007.2103
- Eby, L.A., Crowder, L.B., 2002. Hypoxia-based habitat compression in the Neuse River Estuary: context-dependent shifts in behavioral avoidance thresholds. *Can. J. Fish. Aquat. Sci.* 59, 952–965. doi:10.1139/f02-067
- Ekau, W., Auel, H., Pörtner, H.-O., Gilbert, D., 2010. Impacts of hypoxia on the structure and processes in pelagic communities (zooplankton, macro-invertebrates and fish). *Biogeosciences* 7.
- Fagergren, D., Criss, A., Christensen, D., 2004. Hood Canal Low Dissolved Oxygen Preliminary Assessment and Corrective Action Plan.
- Froehlich, H. E., Roberts, S. B., Essington, T. E. (2014). Hypoxia-inducible factor-1 α mRNA expression in Pacific herring *Clupea pallasii*: figshare.<http://dx.doi.org/10.6084/m9.figshare.1190951>
- Gelman, A., Hill, J., 2010. *Data Analysis Using Regression and Multilevel/Hierarchical Models*, 9th ed. Cambridge University Press, New York.
- Gilly, W. F., Beman, J.M., Litvin, S.Y., Robison, B.H. 2013. Oceanographic and Biological Effects of Shoaling of the Oxygen Minimum Zone. *Ann. Rev. of Mar. Sci.* 5, 393–420. doi:10.1146/annurev-marine-120710-100849.
- Gorr, T.A., Wichmann, D., Hu, J., Hermes-Lima, M., Welker, A.F., Terwilliger, N., Wren, J.F., Viney, M., Morris, S., Nilsson, G.E., Deten, A., Soliz, J., Gassmann, M., 2010. Hypoxia Tolerance in Animals: Biology and Application. *Physiol. Biochem. Zool.* 83, 733–752.
- Haegle, C.W., Schweigert, J.F., 1985. Distribution and characteristics of herring spawning grounds and description of spawning behavior. *Can J Fish Aquat Sci* 42, 39–55.
- Horppila, J., Malinen, T., Nurminen, L., Tallberg, P., Vinni, M., 2000. A metalimnetic oxygen minimum indirectly contributing to the low biomass of cladocerans in Lake Hiidenvesi - a diurnal study on the refuge effect. *Hydrobiologia* 436, 81–90. doi:10.1023/a:1026594006856

- Kodama, K., Rahman, M.S., Horiguchi, T., Thomas, P., 2012a. Upregulation of hypoxia-inducible factor (HIF)-1 alpha and HIF-2 alpha mRNA levels in dragonet *Callionymus valenciennei* exposed to environmental hypoxia in Tokyo Bay. *Mar. Pollut. Bull.* 64, 1339–1347. doi:10.1016/j.marpolbul.2012.05.002
- Kodama, K., Rahman, S., Horiguchi, T., Thomas, P., 2012b. Assessment of hypoxia-inducible factor-1 alpha mRNA expression in mantis shrimp as a biomarker of environmental hypoxia exposure. *Biol. Lett.* 8, 278–281. doi:10.1098/rsbl.2011.0887
- Law, S.H.W., Wu, R.S.S., Ng, P.K.S., Yu, R.M.K., Kong, R.Y.C., 2006. Cloning and expression analysis of two distinct HIF-alpha isoforms - gcHIF-1alpha and gcHIF-4alpha - from the hypoxia-tolerant grass carp, *Ctenopharyngodon idellus*. *Bmc Mol. Biol.* 7. doi:10.1186/1471-2199-7-15
- Levin, L.A., Ekau, W., Gooday, A.J., Jorissen, F., Middelburg, J.J., Naqvi, S.W.A., Neira, C., Rabalais, N.N., Zhang, J., 2009. Effects of natural and human-induced hypoxia on coastal benthos. *Biogeosciences* 6, 2063–2098. doi:10.5194/bg-6-2063-2009
- Longhurst, A.R., 1967. Vertical distribution of zooplankton in relation to the eastern Pacific oxygen minimum. Presented at the Deep Sea Research and Oceanographic Abstracts, Elsevier, pp. 51–63.
- Ludsin, S.A., Zhang, X., Brandt, S.B., Roman, M.R., Boicourt, W.C., Mason, D.M., Costantini, M., 2009. Hypoxia-avoidance by planktivorous fish in Chesapeake Bay: Implications for food web interactions and fish recruitment. *J. Exp. Mar. Biol. Ecol.* 381, S121–S131. doi:10.1016/j.jembe.2009.07.016
- McGinnis, S., Madden, T.L., 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res.* 32, W20–W25.
- Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T.L., Agarwala, R., Schaffer, A.A., 2008. Database indexing for production MegaBLAST searches. *Bioinformatics* 24, 1757–64.
- Newton, J.A., Thomson, A.L., Eisner, L.B., Hannach, G.A., Albertson, S.L., 1995. Dissolved oxygen concentrations in Hood Canal: are conditions different than forty years ago. *Puget Sound Res. '95 Proc. Puget Sound Water Qual. Auth. Olymp. WA* 1002–1008.
- Newton, J., Bassin, C., Devol, A., Kawase, M., Ruef, W., Warner, M., Hannafious, D., Rose, R., 2007. Hypoxia in Hood Canal: An overview of status and contributing factors, Georgia Basin Puget Sound Research Conference. Vancouver, British Columbia.
- Nikinmaa, M., Rees, B.B., 2005. Oxygen-dependent gene expression in fishes. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* 288, R1079–R1090. doi:10.1152/ajpregu.00626.2004
- Nilsson, G.E., Ostlund-Nilsson, S., 2008. Does size matter for hypoxia tolerance in fish? *Biol. Rev.* 83, 173–189. doi:10.1111/j.1469-185X.2008.00038.x
- Parker-Stetter, S.L., Horne, J.K., 2009. Nekton distribution and midwater hypoxia: A seasonal, diel prey refuge? *Estuar. Coast. Shelf Sci.* 81, 13–18. doi:10.1016/j.ecss.2008.09.021
- Parker-Stetter, S.L., Horne, J.K., Langness, M.M., 2009. The influence of midwater hypoxia on nekton vertical migration. *Ices J. Mar. Sci.* 66, 1296–1302. doi:10.1093/icesjms/fsp006
- Pollock, M.S., Clarke, L.M.J., Dubé, M.G., 2007. The effects of hypoxia on fishes: from ecological relevance to physiological effects. *Environ. Rev.* 15, 1–14. doi:10.1139/a06-006
- Prince, E.D., Goodyear, C.P., 2006. Hypoxia-based habitat compression of tropical pelagic fishes. *Fish. Oceanogr.* 15, 451–464.
- Rahman, M.S., Thomas, P., 2007. Molecular cloning, characterization and expression of two hypoxia-inducible factor alpha subunits, HIF-1[alpha] and HIF-2[alpha], in a hypoxia-

- tolerant marine teleost, Atlantic croaker (*Micropogonias undulatus*). *Gene* 396, 273–282. doi:10.1016/j.gene.2007.03.009
- R Core Team, 2013. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna Austria.
- Riedel, B., Pados, T., Pretterebner, K., Schiemer, L., Steckbauer, A., Haselmair, A., Zuschin, M., Stachowitsch, M., 2014. Effect of hypoxia and anoxia on invertebrate behaviour: ecological perspectives from species to community level. *Biogeosciences* 11, 1491–1518. doi:10.5194/bg-11-1491-014
- Rimoldi, S., Terova, G., Ceccuzzi, P., Marelli, S., Antonini, M., Saroglia, M., 2012. HIF-1 α mRNA levels in Eurasian perch (*Perca fluviatilis*) exposed to acute and chronic hypoxia. *Mol. Biol. Rep.* 39, 4009–4015. doi:10.1007/s11033-011-1181-8
- Roberts, J.J., Greco, P.A., Ludsins, S.A., Pothoven, S.A., Vanderploeg, H.A., Hook, T.O., 2012. Evidence of hypoxic foraging forays by yellow perch (*Perca flavescens*) and potential consequences for prey consumption. *Freshw. Biol.* 57, 922–937. doi:10.1111/j.1365-2427.2012.02753.x
- Roberts, S.B., Hauser, L., Seeb, L.W., Seeb, J.E., 2012. Development of Genomic Resources for Pacific Herring through Targeted Transcriptome Pyrosequencing. *PLoS One* 7. doi:10.1371/journal.pone.0030908
- Sagasti, A., Schaffner, L.C., Duffy, J.E., 2001. Effects of periodic hypoxia on mortality, feeding and predation in an estuarine epifaunal community. *J. Exp. Mar. Biol. Ecol.* 258, 257–283. doi:10.1016/s0022-0981(01)00220-9
- Shen, R.-J., Jiang, X.-Y., Pu, J.-W., Zou, S.-M., 2010. HIF-1 α and -2 α genes in a hypoxia-sensitive teleost species *Megalobrama amblycephala*: cDNA cloning, expression and different responses to hypoxia. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 157, 273–280. doi:10.1016/j.cbpb.2010.06.013
- Stramma, L., Schmidtke, S., Levin, L.A., Johnson, G.C., 2010. Ocean oxygen minima expansions and their biological impacts. *Deep-Sea Res. Part -Oceanogr. Res. Pap.* 57, 587–595. doi:10.1016/j.dsr.2010.01.005
- Taylor, J.C., Rand, P.S., Jenkins, J., 2007. Swimming behavior of juvenile anchovies (*Anchoa spp.*) in an episodically hypoxic estuary: implications for individual energetics and trophic dynamics. *Mar. Biol.* 152, 939–957. doi:10.1007/s00227-007-0745-6
- Terova, G., Rimoldi, S., Corà, S., Bernardini, G., Gornati, R., Saroglia, M., 2008. Acute and chronic hypoxia affects HIF-1 α mRNA levels in sea bass (*Dicentrarchus labrax*). *Aquaculture* 279, 150–159. doi:10.1016/j.aquaculture.2008.03.041
- Thomas, P., Rahman, M.S., 2009. Biomarkers of hypoxia exposure and reproductive function in Atlantic croaker: A review with some preliminary findings from the northern Gulf of Mexico hypoxic zone. *J. Exp. Mar. Biol. Ecol.* 381, S38–S50. doi:10.1016/j.jembe.2009.07.008
- Thomas, P., Rahman, M.S., Khan, I.A., Kummer, J.A., 2007. Widespread endocrine disruption and reproductive impairment in an estuarine fish population exposed to seasonal hypoxia. *Proc. R. Soc. B Biol. Sci.* 274, 2693–2702. doi:10.1098/rspb.2007.0921
- Vanderploeg, H.A., Ludsins, S.A., Cavaletto, J.F., Höök, T.O., Pothoven, S.A., Brandt, S.B., Liebig, J.R., Lang, G.A., 2009a. Hypoxic zones as habitat for zooplankton in Lake Erie: Refuges from predation or exclusion zones? *J. Exp. Mar. Biol. Ecol.* 381, S108–S120. doi:10.1016/j.jembe.2009.07.015

- Vanderploeg, H.A., Ludsin, S.A., Ruberg, S.A., Höök, T.O., Pothoven, S.A., Brandt, S.B., Lang, G.A., Liebig, J.R., Cavaletto, J.F., 2009b. Hypoxia affects spatial distributions and overlap of pelagic fish, zooplankton, and phytoplankton in Lake Erie. *J. Exp. Mar. Biol. Ecol.* 381, S92–S107.
- Wang, X.H., Hong, H.S., Mu, J.L., Lin, J.Q., Wang, S.H., 2008. Polycyclic aromatic hydrocarbon (PAH) metabolites in marine fishes as a specific biomarker to indicate PAH pollution in the marine coastal environment. *J. Environ. Sci. Health: Part A* 43, 219–226. doi:10.1080/10934520701792662
- Wenger, R.H., 2006. Mitochondria: oxygen sinks rather than sensors? *Med. Hypotheses* 66, 380–383.
- Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. *Mar. Pollut. Bull.* 45, 35–45. doi:10.1016/s0025-326x(02)00061-9
- Zhang, H., Ludsin, S.A., Mason, D.M., Adamack, A.T., Brandt, S.B., Zhang, X., Kimmel, D.G., Roman, M.R., Boicourt, W.C., 2009. Hypoxia-driven changes in the behavior and spatial distribution of pelagic fish and mesozooplankton in the northern Gulf of Mexico. *J. Exp. Mar. Biol. Ecol.* 381, S80–S91. doi:10.1016/j.jembe.2009.07.014
- Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* 7, 203–214.
- Zhang, Z., Wells, M.C., Boswell, M.G., Beldorth, I., Kirk, L.M., Wang, Y., Wang, Markita Savage, Y., Walter, R.B., Booth, R.E. 2012. Identification of Robust Hypoxia Biomarker Candidates from Fin of Medaka (*Oryzias Latipes*). *Comp. Biochem. and Physio. Part C* 155, 11–17. doi:10.1016/j.cbpc.2011.05.015.
- Zhao, S., Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J Comput Biol* 12, 1045–62.