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A novel and versatile flash-freezing approach for evaluating the health of Delta Smelt

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ABSTRACT

A common approach used to assess environmental impacts in aquatic environments is to measure indicators of stress (biomarkers) and condition of fish within ecosystems. Particularly in estuarine ecosystems with multiple stressors, it is often desirable to quantify a suite of biological endpoints that (1) reflect fish condition at several levels of biological organization and time scales and (2) are sensitive to a range of environmental stressors. However, established methods of preservation and processing of fish for specific endpoints are often incompatible. Here, we developed a novel flash-freezing approach for assessing the health of a small, sensitive fish, the endangered Delta Smelt (*Hypomesus transpacificus*) after collections from the San Francisco Estuary (SFE). We assess whether flash-freezing the entire fish ensures effective preservation of multiple tissues for subsequent biomarker analyses by comparing measurements of fresh to frozen tissue. Tissues included brain, gill, and liver for enzyme activity, kidney and spleen for pathogens, and gills, liver, and gonads for histopathology and reproduction. Although flash-freezing in liquid nitrogen altered the length, weight, and condition factor of Delta Smelt, the percent changes were small (<1.5%). Histological analyses of the cellular morphology of gills, liver, and gonads were similar between both methods. Freezing artefacts were observed in ovaries, but did not hinder the identification and interpretation of cell types and oocyte stages. Freezing did not alter bacterial isolation or the activities of ethoxyresorufin-0-deethylase (EROD) or acetylcholinesterase (AChE), but had a small, negative influence on sodium potassium adenosine triphosphatase (ATPase) activity. Thus, flash-freezing in the field is a versatile preservation method for Delta Smelt, allowing for multiple tissue collections and bioassays from an individual tiny fish exposed to a wide range of natural and anthropogenic stressors. Similar methodology may be applicable to other species for which a range of biological endpoints and histopathology data are needed.

1. Introduction

In the past few decades, researchers have generally used the condition of a piscivorous fish as an indicator of aquatic ecosystem health. Typically, these species are of economic importance, easily collected, and relatively tolerant to a wide range of natural and anthropogenic stressors. The driving force for initiating most fish condition assessments is often related to water quality impairment caused by contaminants from chronic pollution or impacts of catastrophic events. For example, Bevelhimer et al. (2014) utilized a wide range of physiological and energetic responses to evaluate the health of fishes near a coal ash spill in east Tennessee. Similarly, Adams et al. (1996) used a suite of biological endpoints to evaluate the health and condition of wild fish in the Pigeon River, Tennessee that received bleached kraft mill effluent (Adams et al., 1996). In the Clinch River/Watts Barr Reservoir, Adams et al. (1999) quantified the enzymatic, reproductive, and histopathological response of fish to contaminant loading, primarily mercury and PCBs, in the Clinch River/Watts Bar Reservoir system. In other cases, fish health assessments may also aim to address factors associated with the onset and development of disease and reproductive abnormality in natural fish populations (e.g., Pierce et al., 1978; Myers et al., 1998; Johnson et al., 1998).

In estuarine ecosystems, where a wider range of potential stressors exist, a broader approach may be needed to assess organism or ecosystem condition. Estuaries are naturally variable in terms

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of physicochemical (e.g., salinity and temperature) and biological (e.g., algal blooms) conditions, adding complexity to any condition assessment. Although using a suite of biomarkers at several levels of biological organization has been proposed as a weight-of-evidence method to assess estuarine organism and ecosystem health (Adams et al., 1989; Huggett et al., 1992), a comprehensive health assessment is impossible with standard preservation methods. Here, we describe an approach for assessing the condition of the Delta Smelt (*Hypomesus transpacificus*), a small (adults 60–80 mm, standard length) estuarine fish endemic to fresh and brackish water habitats in the San Francisco Estuary (SFE). The SFE is formed by the convergence of the Sacramento and San Joaquin Rivers and the Pacific Ocean (Moyle et al., 1992; Moyle, 2002). The abundance of Delta Smelt has been in decline for decades, leading to the listing of the species as threatened and endangered under the Federal and California State Threatened and Endangered Species Acts, respectively (USFWS, 1993; CDFW, 2014). The SFE is highly altered by a wide range of temporally and spatially covarying anthropogenic impacts. Therefore, a comprehensive condition assessment must be sensitive to many potential stressors, including poor nutrition, contaminants, disease, and changing physicochemical conditions. In addition, Delta Smelt is notoriously sensitive (Moyle et al., 1992), so an effective and rapid preservation method is essential to minimize artefacts caused by sampling stress. Importantly, the threatened and endangered status of the Delta Smelt requires mandatory restrictions on all collections, hence the data gathered from each individual must be maximized.

To address these issues, we developed a method in which Delta Smelt collected in the field are frozen in liquid nitrogen immediately following capture. Then, using staggered, rapid necropsies of fish as they defrost in the laboratory, multiple tissues are processed for measurement of a large suite of biological endpoints. These endpoints include general body and organ condition indices, nutritional indicators, otolith aging and microchemistry, enzymatic markers of exposure, pathogens, histopathologic markers, and reproductive endpoints. However, enzymatic, microbiological, histopathological and reproductive indices are traditionally evaluated using fresh samples. Accordingly, the purpose of this paper is threefold: (1) explain the relevance of flash-freezing for preserving smelt and use in various biomarkers at multiple levels of biological organization, (2) describe the method, and (3) evaluate the compatibility of flash-frozen tissues with endpoints routinely assessed by conventional techniques. For the latter, flash-frozen tissues were evaluated in parallel with fresh tissues to determine the diagnostic accuracy and validity of the non-conventional flash-freezing method.

2. Materials and methods

2.1. Comparison of conventional and flash-freezing methods

Adult Delta Smelt from the Fish Conservation and Culture Laboratory (FCCL) in Tracy, California were used for comparison of endpoints from fresh and flash-frozen tissues. The fish were transported live to the Aquatic Health Program at UC Davis, California. The influence of flash-freezing was evaluated using 95 fish: 10 for morphometry, 10 for enzyme activity (gill, liver, and brain), 10 for pathogens (spleen and kidney), 60 for histology (gill, liver, and gonad), and 5 for oocytes (size and internal structure).

For morphometry, enzyme, and pathogen endpoints as described below, groups of fish (numbers are indicated above) were processed where for each fish, one portion of tissue is fresh and a corresponding portion is flash-frozen. All endpoints including histology were performed and analyzed independently as needed and as described below. In this context, the results for each endpoint from each fish must be only attributed to the differences between fresh and frozen tissues to determine the compatibility of flash freezing to methods that traditionally use fresh samples (i.e., morphometry, enzyme, pathogen, histology).

In cases for which a significant effect of freezing was found, we used simple linear regression to develop a relationship between fresh and frozen measurements.

2.2. Morphometric analysis

Individual Delta Smelt ($n = 10$) were pithed, blotted dry gently on paper towels, measured to the nearest millimeter (mm) for fork length and weighed to the nearest gram (g) for body weight. Fulton's condition index (K) was calculated using $K = 10^5\ W/L^3$ where W is the weight of the fish in g, L is the length of the fish in mm. After these measurements, each fish was wrapped in aluminum foil, labeled accordingly, and then flash-frozen in liquid nitrogen. After 10 days, the fish were removed, thawed briefly, and blotted dry as above prior to measurements of fork length and body weight for condition factor calculation. Differences in morphometric features were compared and analyzed between fresh and flash-frozen tissues using paired t-tests.

2.3. Enzyme analysis

Enzyme activity was compared between fresh and flash-frozen brain, gill, and liver of Delta Smelt ($n = 10$). The fish were pithed, the tissues were excised, and each organ was cut into 2 portions. One portion of each organ was analyzed immediately (without freezing) for enzyme activity. The second portion was kept in a microcentrifuge tube and flash-frozen in liquid nitrogen and stored in a −80 °C freezer for one week before conducting the analysis. The brain was analyzed for acetylcholinesterase (AChE, $n = 10$), the liver for ethoxyresorufin-0-deethylase (EROD, $n = 10$), and the gills for sodium potassium adenosine triphosphatase (ATPase) activity ($n = 9$; gill tissue from one fish was accidentally lost). All of the enzymatic assays followed published methods with minor modifications: AChE (Ellman et al., 1961), EROD (Eggens and Galgani, 1992; Burke and Mayer, 1974), and ATPase (Holliday, 1985). The enzymatic activities were normalized to protein concentration in soluble fraction using the Lowry protein assay (Lowry et al., 1951) prior to determination of enzyme levels. Statistical differences in enzymatic activities were compared between fresh and flash-frozen tissues using paired t-tests.

2.4. Bacterial and viral isolation

Isolation of bacteria and viruses were compared between fresh and frozen tissue using pooled spleen and kidney from individual Delta Smelt ($n = 10$). Tissues were processed as follows: the kidney and spleen were excised from each fish, pooled, suspended in 350 μl Minimum Essential Medium Eagle (MEM, Sigma–Aldrich) in a microfuge tube, and homogenized for 1 min using a sterile pellet pestle (Kimble-Chase Kontes, Fisher Scientific). After thorough mixing, a 50 μl homogenate (fresh sample, conventional method) was inoculated onto each of the three culture plates as indicated below. The remaining half of each homogenate of pooled tissue was stored in liquid nitrogen for 7 days (freezing method). Frozen samples were thawed on ice prior to inoculation onto each medium.

For bacterial isolation, subsamples of the fresh or frozen homogenate were inoculated (ca. 20 μl/plate) onto three separate culture plates: Blood agar for general bacterial isolation, Tryptone Yeast Extract Salt (TYES) for fastidious flexibacteria, and Middlebrook 7H10 agar (Difco) for mycobacteria. The plates were incubated at 15 °C for 7 days and examined for bacterial growth. Bacterial isolation followed standard procedures (AFS-Fish Health