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4 1 **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-O-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.

Keywords: Flash-freezing, Necropsy, Biomarker, Delta Smelt, San Francisco Estuary

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 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) was pithed, blotted dry gently on paper towels, measured to the nearest millimeter (mm) for fork length and weighed to the nearest grams (g) for body weight. Fulton's condition index (K) was calculated using $K = 10^5 \cdot W/L^3$ 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-O-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, Fishewr 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 Section, 2007). The severity of anomaly was assessed according to Goede and Barton (1990) where values were assigned (0–4) based on the number and identity of colonies that grew on plates, referred to as “pathogen score”: 0 = zero colonies, 1 = 1–10 colonies, 2 = 11–20 colonies, 3 = 21–200 colonies, and 4 ≥ 201 colonies. After bacterial inoculation, the supernatant from the remaining homogenate from each of the fish was used for virus isolation by tissue culture following standard methods (AFS – Fish Health Section, 2007).

Colonies on Blood agar, TYES, and MiddleBrook 7H10 agar were examined for basic morphologic and biochemical characteristics (AFS – Fish Health Section 2007). Dominant bacterial isolates were identified presumptively by phenotypic characterization and verified by sequencing using the 16S ribosomal RNA (rRNA) gene (Cunningham, 2002). The universal bacterial primers EUBA/ EUBB were used for PCR to amplify the 16S ribosomal DNA (rDNA) regions. The amplified products were submitted to Davis Sequencing Inc. (<http://www.davissequencing.com/index.htm>) to determine the DNA sequences of representative isolates and then compared to known sequences in the GenBank database.

For virus isolation, cell lines including EPC (*Epithelioma papulosum* Cyprinid carp), CHSE– 214 (Chinook Salmon Embryo), and BF2 (Bluegill Fry) were inoculated with each supernatant sample. In addition, specific cell lines that we developed from Delta Smelt gills and threadfin shad gills following standard methods (AFS – Fish Health Section, 2007) were also used for virus isolation. Cell cultures were incubated at 15°C for a minimum of 21 days and subcultured within 14 days.

2.5. Histological analysis

Adult Delta Smelt (n=60) from FCCL of the same age were assigned randomly to conventional and flash-freezing methods. In the conventional method, individual whole fish (n=30) were fixed in 10% neutral buffered formalin for one week. At the end of the week, necropsy was performed during which gills, liver, and gonads were removed and placed in a tissue cassette in 10% neutral buffered formalin for one week. For the flash-freezing method (n=30), individual fish wrapped in aluminum foil were placed in liquid nitrogen for one week. After one week, necropsies were performed during which the gills, liver, and gonads were excised from each fish, placed into tissue cassettes, and fixed in 10% neutral buffered formalin for one week. The tissues from both methods (fresh fixed vs. frozen fixed) were then dehydrated in graded ethanol series, embedded in paraffin, sectioned at 3 µm, stained with Hematoxylin & Eosin (H&E), dried, and coverslipped (for more detailed methods see Teh et al., 1997). Fresh and frozen tissues were examined using essential criteria for analyzing histologic features including: 1) tissue architecture and integrity, 2) fixation quality, 3) cellular detail and resolution such as appearance of nuclei, nucleoli, and cell membranes, and 4) staining quality of tissues using a BH–2 Olympus microscope. In addition, one whole wild juvenile Delta Smelt was collected and processed to demonstrate the applicability of the flash-freezing method to a Delta Smelt collected and frozen in the field (for further examples of histopathology of flash-frozen wild fish see Hammock et al. 2015).

2.5.1. Oocyte morphology in histological sections

Oocyte structure was assessed in fresh and frozen ovarian sections to determine differences in oocyte shape, integrity of cortical alveoli, and appearance of egg yolk bodies at the

vitellogenic stage. Earlier descriptions of oocyte stages in Delta Smelt were followed according to Mager (1996) and Kurobe et al. (*in revision*)

2.6. Oocyte size

The influence of flash-freezing on size and structure of oocytes was evaluated using ovaries from 5 reproductively mature females. From each fish, masses of oocytes were removed, placed on glass slides (25–59 oocytes/fish), and individual oocytes were photographed. The slides were immediately placed in conical tubes and flash-frozen in liquid nitrogen. Two hours later, the conical tubes were removed and a second photo was taken of each oocyte after defrosting at room temperature. The size of individual oocytes (n=199 from the 5 fish) before and after the cryopreservation in liquid nitrogen was measured using ImageJ software (<http://rsbweb.nih.gov/ij/>) by setting a micrometer scale, tracing the oocyte, and then calculating its area using a computational equation in the software. The mean size of the oocytes before and after freezing was compared using a paired t-test.

2.7. Application of flash-freezing for preserving Delta Smelt collected in the field

This section describes the use of flash-freezing for preserving smelt following collection in the field and then evaluated for 8 different biological endpoints in the laboratory. While the results of these endpoints are not discussed in this paper, the description aims to emphasize the rationale for using flash-freezing for preserving large numbers of fish collected across multiple sites. During trawl sampling in the San Francisco Estuary (SFE), fish that are captured across locations and identified as Delta Smelt are individually wrapped with aluminum foil, assigned an ID number, and placed in liquid nitrogen immediately. The entire process (identification, wrapping, and freezing) typically takes 5 min, although it can take up to 15 min if many fish are caught or field conditions are difficult. Delta Smelt are kept in liquid nitrogen continuously until the fish are processed at the Aquatic Health Program at the University of California, Davis using staggered necropsies. During necropsies, fish are removed from liquid nitrogen, fork length (FL) and body weight (BW) are measured, and then multiple organs are immediately excised and preserved for 8 different biological endpoints: 1) otolith for aging and microchemistry, 2) fins for genetic analysis, 3) brain, gill, and liver in liquid nitrogen for enzyme analysis, 4) muscle in liquid nitrogen for nutritional analysis, 5) kidney and spleen in buffered solution for pathogen

analysis, 6) gills, liver, and gonads in fixative for histology and reproduction, 7) gastrointestinal tract for gut content analysis, and 8) carcass for body burden analysis. The entire necropsy procedure takes 5–10 min per fish. Of these endpoints, the methods for analyzing enzyme, pathogens, and histology using flash–frozen tissues are novel or unconventional. Hence, the equivalence of these methods was evaluated in the current study by comparing measurements made from fresh to flash–frozen samples using hatchery raised Delta Smelt, as described above. To demonstrate the suitability of the freezing method for field samples, photographs of one Delta Smelt that was collected and flash–frozen in the SFE and processed in the lab for histopathology was included.

3. Results

3.1. Morphometry

Fork length, body weight and condition factor of fresh and flash–frozen Delta Smelt are shown in Table 1. Paired t-tests showed that there were significant differences for fork length ($df=9$, $P=0.004$), body weight ($df=9$, $P=0.003$), and condition factor ($df=9$, $P=0.02$). On average, the percent changes of fork length was 0.55% and body weight was 0.21% lower in flash–frozen than in fresh tissues while condition factor was 1.5% higher in flash frozen than in fresh tissues (Table 1). We found strong, linear relationships between frozen and fresh in fork length (linear regression, $df=8$, $P<0.001$, $R^2=0.99$), body weight (linear regression, $df=8$, $P<0.001$, $R^2=0.99$), and condition factor (linear regression, $df=8$, $P<0.001$, $R^2=0.97$). The regression equation for fork length, body weight, and condition factor are $Fresh = 2.558 + 0.972Frozen$ (fork length), $Fresh = 0.00955 + 0.999Frozen$ (body weight), $Fresh = 0.0416 + 0.933Frozen$ (condition factor), where *Fresh* is measurement in fresh tissue and *Frozen* is measurement in frozen tissue.

3.2. Enzyme activities

Enzymatic activities of fresh and flash–frozen tissues for brain acetylcholinesterase (AChE), liver ethoxyresorufin-0-deethylase (EROD), and gill sodium-potassium adenosinetriphosphatase (ATPase) activity are shown in Table 2. There were no significant differences in AChE (paired t-test, $df=9$, $P=0.15$) and EROD (paired t-test, $df=9$, $P=0.86$) activities between fresh and flash–frozen tissue. However, ATPase activity was 13.9% lower in flash–frozen than in fresh tissues (Table 1, paired t-test, $df=8$, $P=0.025$). We found a strong,

linear relationship between frozen and fresh ATPase activity (linear regression, $df=7$, $P=0.0025$, $R^2=0.85$). The regression equation is $Fresh = 0.5336 + 0.9734Frozen$, where *Fresh* is ATPase activity measured in fresh tissue and *Frozen* is ATPase activity measured in frozen tissue.

3.3. Pathogen isolation

The types and number of bacterial colonies, and overall pathogen scores were similar between fresh and 7-day post frozen tissues after incubation at 15°C for 7 days (Table 3). Based on the frequency of occurrence of these bacteria in fresh and frozen tissues, the pathogen scores for all 10 fish were equal between the two treatments: four fish received a pathogen score of 1 in both treatments while six fish received a score of 0 in both treatments. The dominant bacteria that were isolated and identified from spleen and kidney pools of either fresh or frozen smelt include *Flavobacterium*, *Aeromonas*, *Pseudomonas*, *Microbacterium*, *Sphingopyxis*, *Zooglea*, and *Bacillus*. The presence of these bacteria in the spleen and kidney of smelt was not altered by freezing. Viral agents were not found in either the fresh or corresponding frozen tissues.

3.4. Histology

Histological demonstrations of a 3 µm thick whole body flash-frozen juvenile smelt (caught in the field) section stained with hematoxylin and eosin (H&E) are shown in Fig. 1A–G. Morphological details indicate that liver, gill, testis and ovary of all 30 fish were sufficiently preserved and allowed reliable pathological diagnostics. The sections (Fig. 1A–G) are free of distortion with clear resolution of each organ (eye, gill, liver, intestine, pancreas, and immature gonad). Higher magnifications show that the blood cells in the blood vessels of all organs are preserved (Fig. 1B–C). Histological comparison of gill, gonad, and liver tissues that were processed by flash-freezing and conventional fixing techniques are shown in Fig. 2–7.

3.4.1. Oocyte morphology in histological sections

Flash-freezing had minimal effects on the internal cellular morphology of oocytes at immature stages (stage 1 and 2, Fig. 6). The presence of nucleolus on the periphery of the nucleus, the characteristic feature of stage 2 oocytes, was not altered (Fig. 7A–B). However, artefacts associated with flash-freezing were observed in the gill, liver, and oocytes at mature stages. These included 1) H&E staining of nucleus and cell membranes of frozen tissues were

not as distinctly demarcated as the fresh fixed tissue and 2) staining intensity of eosin on erythrocytes in flash-frozen fixed tissues were lighter than that of fresh-fixed tissues (Fig. 2), and 3) separation of interstitial space in gill and 4) intracytoplasmic vacuoles in late stage 3 and 4 oocytes (Fig. 4B, 5B1). At stage 3 late, cortical alveoli ruptured and merged together, forming a transparent large mass located in the center of oocytes dislocating the nucleus toward the periphery (Fig. 6B). Egg yolk bodies at the vitellogenic stage (stage 4) were also ruptured by freezing, leaving traces of yolk bodies in oocytes (Fig. 7 C–D). Freezing also affected the shape of oocytes, especially at the vitellogenic stage; the chorion layer of the oocyte at stage 4 late exhibited an irregular undulating shape in the flash-freezing treatment whereas oocytes from the fresh fish showed an oval or circular shape (Fig. 6B).

3.4. 2. Oocyte size

Flash-freezing had a small but significant effect on the size of oocytes (paired t-test, $df=198$, $P<0.0001$). The mean area of frozen oocytes ($n=199$ from 5 fish) was 2% larger than fresh oocytes. There was a strong, linear relationship between fresh and frozen oocyte area (linear regression, $df=197$, $P<0.0001$, $R^2=0.75$). The regression equation is $Fresh = 0.10308 + 0.82481Frozen$, where *Fresh* is oocyte area determined from fresh tissue while *Frozen* is oocyte area determined from frozen tissue.

4. Discussion

The San Francisco Estuary is influenced by many covarying environmental impacts. The combined effects of multiple stressors are thought to be causing the general decline of fish abundance in the estuary, particularly the Delta Smelt (e.g. Moyle et al. 1992, Bennett 2005, Sommer et al. 2007). Gaining a better understanding of the causes for the decline of Delta Smelt necessitates a spatially explicit and comprehensive understanding of the health and condition of fish across multiple levels of biological organization and time scales. Moreover, the indices must be sensitive to the many stressors that could be causing the decline such as poor nutrition, contaminants, changing water quality, and disease (Sommer et al. 2007). The current study describes and validates the use of flash-freezing to allow multiple, previously incompatible, indices to be measured on the same fish. However, because freezing fish tissue is not a standard procedure for several of the biomarkers and other endpoints, our study focused on determining

the compatibility of flash-frozen tissues of Delta Smelt when used with conventional methods for assessing health and condition of fish.

Freezing and fixing in formalin can reduce fish length and weight, however the effect of freezing is minimal compared to formalin preservation (Ogle 2009; Ajah and Nunoo 2003). In this study, although flash-freezing in liquid nitrogen altered the length, weight, and condition factor of Delta Smelt, the percent changes were small. Furthermore, strong linear relationships were observed between fresh and frozen fish in terms of length, weight, and condition factor, providing a method to convert between frozen and fresh measurements.

Flash-freezing of entire Delta Smelt in liquid nitrogen did not affect the activities of EROD in the liver and AChE in the brain, but it did reduce ATPase activity in flash-frozen gill tissues. As ATPase is a membrane bioassay, flash-freezing and thawing may have disrupted the membrane integrity of gills, resulting in lower ATPase activity due to freezing artefacts as shown in Fig. 5B1. However, there was a strong linear relationship between fresh and frozen ATPase activity, providing a method to convert between frozen and fresh measurements. Thus, flash-freezing did not impair our ability to measure the activities of these enzymes.

Using frozen tissues also demonstrated suitable outcomes for isolation of pathogens that commonly use fresh tissues. Bacterial organisms were viable in frozen smelt tissues, and pathogen scores were similar between fresh and flash-frozen tissues. These results suggest that flash-freezing is an appropriate method for preserving field samples of Delta Smelt, (present results, Baxa et al. 2015) to minimize the deterioration of tissues for microbial isolation. Bacterial isolation was compared between fresh and frozen tissues as the standard method requires the use of fresh tissue samples (AFS – Fish Health Section, 2007). Under extenuating circumstances such as in field monitoring surveys, freezing samples can be used to preserve the integrity of fish tissues for isolation of microbial organisms (Noga, 1996; Klinger and Francis–Floyd, 2013). Moreover, our results showed that the bacteria that were recovered in frozen and fresh tissues of smelt were similar in terms of species type (present results; Baxa et al., 2015). Our findings corroborate previous results in other fish species where common bacterial fish pathogens remained viable at 20–60 days post freezing (Brady and Vinitnantharat, 1990). For viral isolation, the World Health Organization (2000) suggests preserving clinical specimens in liquid nitrogen or in –20 or –70°C. The absence of viral agents from parallel samples of fresh and frozen tissues of Delta Smelt indicates that viruses were not detectable in either tissue type.

Overall, we detected no difference in pathogen types and scores between fresh and frozen smelt samples.

Our study demonstrates the advantage of freezing smelt prior to fixation for histological analysis, improving its compatibility with analyses of other biomarkers and endpoints. Although directly preserving the fish in fixative after collection provides standard tissues for histopathology, fixed tissues are no longer useful for the other measurements. For example, formalin-fixed smelt are ideal specimens for evaluating histopathological changes but will no longer be useful for the analyses of enzymatic activities, pathogen isolation, otolith microchemistry, RNA/DNA ratio, and triglycerides. One of the biggest challenges for utilizing flash-frozen tissues is the necessity for histopathologists to be familiarized on evaluating and interpreting lesions and other microscopic changes from frozen samples. Flash-frozen tissues of Delta Smelt revealed good histologic characteristics in gill, gonad, and liver sections based on fundamental criteria, including 1) tissue architecture and integrity, 2) fixation quality, 3) cellular detail and resolution such as appearance of nuclei, nucleoli, and cell membranes, and 4) staining quality of tissues (Fig. 2–4). Despite the cellular changes or tissue artefacts arising from flash-freezing, the key histologic features were observed in frozen sections that allowed us to detect and to distinguish actual alterations such as intracytoplasmic vacuoles in mature oocytes (Fig. 4B) and separation of ionocytes from the interstitial tissue at the base of the primary lamella (Fig. 5B1). These results suggest that flash-freezing whole fish and then preserving in formalin for histopathologic analysis can provide critical information regarding microscopic changes or lesions in Delta Smelt that may develop as an indicator of exposure to stressors as demonstrated in Hammock et al. (2015).

Flash-freezing did not alter the cellular morphology of oocytes at immature stages 1, 2 and early stage 3. However, cryopreservation ruptured the cortical alveoli and egg yolk bodies in later oocyte stages. Oocytes in late stage 3 have the most abundant cortical alveoli that occupy a large area of the oocyte. Ruptured cortical alveoli formed a large mass of cortical alveolus at the center of the oocyte, dislocating the nucleus toward the periphery. Likewise, freezing disrupted the integrity of yolk bodies in stage 4 oocytes. Ruptured yolk bodies merged with each other or with the cytoplasm and lost their circular shape. Despite the freezing artefacts observed in late stage 3 to 4, the maturity level of DS oocytes could be accurately assessed from frozen Delta Smelt. Oocytes at late stage 3 and early stage 4 can be distinguished based on the presence or

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4 379 absence of egg yolk bodies. To avoid misclassification of maturity stages, we additionally
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6 380 measured oocyte dimensions that help to identify specific stages of oocyte development. We
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8 381 have established ranges of oocyte sizes in each developmental stage. Though statistically
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10 382 significant, there was a strong linear relationship between fresh and frozen oocyte area
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12 383 measurements, so the linear model fit to the frozen and fresh oocyte area data can be used to
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14 384 convert between frozen and fresh measurements.

15 385 The fundamental goal of employing flash-freezing is to quickly and effectively preserve
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17 386 fish until tissues can be processed for multiple endpoints that are useful indicators of fish health
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19 387 and condition. The advantages of flash-freezing smelt samples are that: 1) a wider range of
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21 388 biomarkers and biological endpoints can be measured on the same fish than with conventional
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23 389 preservation techniques allowing for evaluation and integration of multiple stressor responses, 2)
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25 390 quick preservation minimizes handling and sampling stress thereby maximizing sample integrity
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27 391 for various sensitive endpoints such as enzymatic activities, RNA/DNA ratio, triglycerides, and
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29 392 liver glycogen, 3) dissections and tissue processing can be done in the lab more precisely and
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31 393 rapidly than in the field, 4) the speed of the preservation method ensures that each fish is treated
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33 394 similarly, even if a large number of fish is sampled in a single net. The combination of these
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35 395 advantages allows us to integrate the results of histopathology and determinations of oocyte
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37 396 maturity, pathogens, and enzymatic activities with other biomarkers and indices. Our findings
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39 397 demonstrate that flash-frozen tissues were either similar (EROD, AChE, pathogens, histology)
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41 398 or comparable via linear relationships (ATPase and oocyte size) to analyses of fresh tissues.
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43 399 Thus, the freezing approach allows for a more comprehensive assessment of health and condition
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45 400 of an individual fish than with fixed tissues that can only offer a limited number of endpoints. In
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47 401 addition, the use of enzyme and immunohistochemistry to localize enzymatic biomarkers (e.g.,
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49 402 Cytochrome P450s, ATPase, and AChE) on target organs of fish considered too small for
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51 403 biochemistry also becomes available. Together, histopathologic and histochemical biomarkers
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53 404 are effective tools for detecting chronic biological effects of environmental contaminants (Teh
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55 405 and Hinton, 1993, Teh et al., 1999; Tay et al., 2003). This study highlights the benefits of fish
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57 406 tissue preservation using flash-freezing to augment the range of data and information that can be
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59 407 generated from an individual fish. Freezing artefacts should not deter a trained histopathologist
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61 408 to distinguish actual cellular morphology. It should be noted that other than an experienced
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63 409 histopathologist, a competent histotechnician plus a standardized method for processing,
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orientation, sectioning, and staining of flash–frozen tissues are highly recommended to minimize artefacts that lead to misdiagnoses of lesions (Wolfe et al. 2014).

It is important to note that the underlying objective of this paper is to evaluate the use of flash freezing for preserving and maximizing the use of multiple tissues for a suite of biological endpoints on a small endangered fish. The fundamental reason for the comparison between frozen and fresh tissues is to demonstrate and validate the feasibility and compatibility of the freezing method to a method which traditionally employs fresh samples such as those required for histopathology, bacterial isolation, enzymatic activities, including basic fish morphometrics such as fork length and gross weight. In particular, cellular and morphologic details are critical features in histopathology that have been adequately confirmed in frozen tissue sections in our study providing a dependable histopathologic evaluation in Delta Smelt.

In summary, the San Francisco Estuary is among the most altered ecosystems worldwide (Glibert 2010, Mount et al. 2012), requiring that condition assessments be sensitive to a suite of potential stressors. By adopting a holistic and versatile method of freezing field samples, a flash–freezing approach can integrate multiple biomarkers and biological endpoints addressing a critical need for information on the impacts of stressors such as contaminants, pathogens, food limitation, and changing physicochemical conditions (e.g., Hammock et al. 2015). Over time, the baseline information based on endpoints generated from individual frozen samples, as examined in the current study, will serve as the groundwork for assessing future changes on the overall health of Delta Smelt in the San Francisco Estuary. Flash-freezing could be used in other fish condition assessments for which a wide range of biological endpoints need to be combined with histopathological analysis.

5. Acknowledgements

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Table 1. Comparison of fork length, body weight, and condition factor in fresh and flash–frozen tissues of Delta Smelt.

Fish	Length		Body Weight		Condition Factor (K)	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
1	85.87	85.56	5.5595	5.5497	0.8780	0.8860
2	71.80	70.74	3.1026	3.0937	0.8382	0.8739
3	84.56	84.16	4.4647	4.4608	0.7384	0.7483
4	76.20	75.71	4.3928	4.3855	0.9928	1.0106
5	76.31	76.14	3.2842	3.2762	0.7391	0.7422
6	77.24	77.21	3.6148	3.6087	0.7844	0.7840
7	72.54	71.56	3.0338	3.0389	0.7948	0.8293
8	77.50	77.04	3.2277	3.2207	0.6934	0.7044
9	71.53	71.39	2.6677	2.6497	0.7289	0.7282
10	76.37	76.22	3.5401	3.5256	0.7947	0.7962
Mean ± SE	76.99±1.54	76.57±1.58	3.69±0.28	3.68±0.28	0.80±0.25	0.81±0.26

Condition factor (K) = $10^5 \cdot \text{Body Weight} / \text{Fork length}^3$

Table 2. Comparison of enzyme activities in fresh and flash–frozen tissues of Delta Smelt. Tissues were flash–frozen in liquid nitrogen and then stored at –80°C for one week prior to enzyme analysis; fresh tissues were analyzed immediately after the fish were sampled.

Fish	Fresh EROD	Frozen EROD	Fresh AChE	Frozen AChE	Fresh ATPase	Frozen ATPase
1	0.155	0.120	14.65	14.99	2.63	2.28
2	0.075	0.068	15.31	14.79	3.97	3.54
3	0.029	0.024	21.35	21.17	2.36	2.15
4	0.037	0.030	20.00	18.30	3.82	2.23
5	0.212	0.194	20.60	17.30	2.63	2.28
6	1.341	1.419	18.45	20.77	5.95	5.14
7	0.025	0.018	14.43	13.25	1.62	1.17
8	0.297	0.321	19.79	19.81	4.57	4.68
9	0.020	0.026	18.27	16.16	2.23	2.19
10	0.158	0.146	26.02	24.82	NA	NA
Mean ± SE	0.235 ± 0.12	0.237 ± 0.13	18.89±1.12	18.14±1.12	3.31±0.41	2.85±0.43

EROD: ethoxyresorufin-O-deethylase (pmoles/mg/min) in liver

AChE: acetylcholinesterase (μmoles/mg/min) in brain

ATPase: sodium (Na⁺)/potassium (K⁺) adenosinetriphosphatase (μmoles/pi/hr/mg protein) in gill

Table 3. Number of bacterial colonies and overall pathogen score from fresh or frozen tissues (pools of kidney and spleen) of Delta Smelt.

Fish number	Blood Agar General isolation		Tryptone Yeast Extract (TYES) Flexibacteria		MiddleBrook 7H10 Mycobacteria		Overall Pathogen Score	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
1	1	1	3	2	0	0	1	1
2	0	0	1	1	0	0	1	1
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	2	2	0	0	1	1
8	0	0	0	0	0	0	0	0
9	0	0	2	2	0	0	1	1
10	0	0	0	0	0	0	0	0

Number of colonies/50 µl of tissue homogenate for each type of culture plate using fresh or frozen tissue, and the overall pathogen score. Pathogen Score: 0=absence of bacterial growth; 1=minimal number of colonies (N=1–10); 2=moderate number of colonies (N=11–20); 3=high number of colonies (>21–200); 4=colonies are too numerous to be counted (>201). Pathogen scores were assigned based on the range of colony numbers that grew on plates.

Fig. 1. Flash-frozen juvenile Delta Smelt from the San Francisco Estuary: A1–A6 show the respective organs indicated in Fig. 1 at higher magnification. (A1) gill showing ionocytes (arrowhead) and proliferation of epithelial cells (arrow). (A2) liver showing a mitotic figure (arrow) and blood cells in sinusoid (arrowhead). (A3) stomach (S), pancreas (P) and immature gonad (IG). (A4) higher magnification of A3 showing the gastric gland (arrow) and copepod (arrowhead) in the stomach. (A5) higher magnification of A3 showing primordial germ cell (arrow) of immature gonad, and (A6) higher magnification of A3 showing pancreas (P) and adipose tissue or fat cell (arrow). The fish was frozen in liquid nitrogen for one week, fixed in 10% neutral buffered formalin for another week, and sections were stained with Hematoxylin and Eosin (H&E).

Fig. 2. Sections of fresh (A) and flash-frozen (B) formalin fixed liver of Delta Smelt. Note that staining characteristic of erythrocyte (arrowheads) and cellular detail of nuclei and nucleoli (arrows) are more prominent in fresh-fixed than flash-frozen fixed liver. The fish was frozen in liquid nitrogen for one week, fixed in 10% neutral buffered formalin for another week, and sections were stained with H&E.

Fig. 3. Fresh (A) and flash-frozen (B) formalin fixed mature Delta Smelt testis. Various stages of spermatogenesis are shown. SG = spermatogonia, SC = spermatocytes, ST = spermatids, SZ = Spermatozoa. The fish was frozen in liquid nitrogen for one week, fixed in 10% neutral buffered formalin for another week, and sections were stained with H&E.

Fig. 4. Fresh (A) and flash-frozen (B) formalin fixed ovary, H&E-stained sections. Various stages of oogenesis are shown. IO = stage 1 and 2 immature oocyte, CA = early cortical alveoli stage 3, VS = vitellogenic stage 4. Note formation of large vacuoles in late stage 3 (*) and stage 4 (**) in Fig. 4B. Individual oocytes were measured before (fresh) and after a 2-hour cryopreservation in liquid nitrogen.

Fig. 5. Fresh (A) and flash-frozen (B) formalin fixed Delta Smelt gill showing the primary (P) and secondary (S) lamellae. A1 and B1 show the respective primary and secondary lamellae indicated in A and B at higher magnification. 1 = undifferentiated basal cell, 2 = epithelial cell, 3 = pillar cell, 4 = ionocyte, and 5 = erythrocyte. Note: arrows point to a noticeable gap between ionocyte and interstitial tissue at the base of primary lamella, a sign of a freezing artefact. The fish was frozen in liquid nitrogen for one week, fixed in 10% neutral buffered formalin for another week. Sections were stained with H&E.

Fig. 6. Developmental stages of Delta Smelt oocytes. **(A):** Oocyte stages from freshly fixed fish without freezing; **(B):** Oocyte stages from flash-frozen fixed fish. The internal structure of Delta Smelt oocytes at immature stages 1 and 2 was not altered by freezing while stages 3 and 4 showed disrupted cortical alveoli and egg yolk bodies **(B)**. The cortical alveoli ruptured and merged together, resulting in dislocation of the nucleus toward the periphery at late stage 3 through mid-stage 4 **(asterisks)**. Freezing also affected the shape of oocytes at the vitellogenic stage: the chorion layer of late Stage 4 oocyte exhibited an irregular, undulating shape whereas oocytes from the fresh fixed fish showed an oval or circular shape. Sections were stained with H&E.

Fig. 7. Internal structure of Delta Smelt oocytes at early stage 2 (**A, B**) and late stage 4 (**C, D**). Note that the 2-hour cryopreservation in liquid nitrogen of individual oocytes (**B, D**) caused the egg yolk bodies to rupture and merge together, leaving traces of yolk bodies in the cytoplasm of the oocytes as indicated by arrows (**D**). The internal structure of early stage 2 (arrows) was not altered before and after cryopreservation in liquid nitrogen (**A, B**). Sections were stained with H&E.

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